03-29-01 JC13 Rec'd PCT/PTO 28 MAR 2001 PCT $

FORM PTO-1390 (Modified) (REV 11-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
33,383-00	
U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	
09/806370	

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US99/22520	September 30, 1999	September 30,1998

TITLE OF INVENTION
MUTANT CHOLERA HOLOTOXIN AS AN ADJUVANT

APPLICANT(S) FOR DO/EO/US
HOLMES, Randall K.; JOBLING, Michael G.; ELDRIDGE, John H.; GREEN, Bruce A. ; HANCOCK, Gerald E.; PEEK, Joel A.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U S.C. 371 (c) (2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made, however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S C. 371(c)(3))
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5))

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1 97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☒ A change of power of attorney and/or address letter.
19. ☐ Certificate of Mailing by Express Mail
20. ☐ Other items or information:

25291
PATENT TRADEMARK OFFICE

CERTIFICATION UNDER 37 C.F.R.1.10

I hereby certify that this paper and the documents referred to as enclosed therein are being deposited with the United States Postal Service on the date written below in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EF320443451 US addressed to the Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

3/28/01
Date

Alan M. Gordon
Alan M. Gordon

U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/806370	PCT/US99/22520	33,383-00

21. The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☐ Neither international preliminary examination fee (37 CFR 1 482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**		
☒ International preliminary examination fee (37 CFR 1 482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO **$840.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1 445(a)(2)) paid to USPTO **$690.00**		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$840.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	27 - 20 =	7	x $18.00	**$126.00**	
Independent claims	12 - 3 =	9	x $78 00	**$702.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
	TOTAL OF ABOVE CALCULATIONS =			**$1,668.00**	
Reduction of 1/2 for filing by small entity, if applicable Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**. ☐				**$0.00**	
	SUBTOTAL =			**$1,668.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
	TOTAL NATIONAL FEE =			**$1,668.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3 31) **(check if applicable).** ☐				**$0.00**	
	TOTAL FEES ENCLOSED =			**$1,668.00**	
				Amount to be: refunded	**$**
				charged	**$**

☐ A check in the amount of to cover the above fees is enclosed.

☒ Please charge my Deposit Account No. **01-1300** in the amount of $1,668.00 to cover the above fees.
A duplicate copy of this sheet is enclosed

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **01-1300** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Alan M. Gordon
Patent Law Department
American Home Products Corporation
Five Giralda Farms
Madison, NJ 07940
Telephone: (845) 732-4636

Alan M. Gordon
SIGNATURE

Alan M. Gordon
NAME

30,637
REGISTRATION NUMBER

3/28/01
DATE

 Docket No: 33,383-00
Patent

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re of Application of:	Randall K. Holmes et al.		
Serial No.:	09/806,370	Group Art No.:	To be Assigned
Filed:	September 30, 1999	Examiner:	To be Assigned
For:	Mutant Cholera Holotoxin as an Adjuvant		
Confirmation No.:	8568		
Customer Number:	25291		

Commissioner for Patents
Washington, DC 20231

October 3, 2001

Sir:

AMENDMENT TRANSMITTAL LETTER

1. Enclosed please find the following documents for the above-identified application:

Preliminary Amendment

CERTIFICATE OF MAILING 37 CFR §1.10

I hereby certify that this paper and the documents referred to as enclosed therein are being deposited with the United States Postal Service on the date written below in an envelope as "Express Mail Post Office to Addressee" Mailing Label NumberET334776318US addressed to the Commissioner for Patents, Washington, DC 20231.

10/3/01
Date

Alan M. Gordon

2. Fee calculation

CLAIMS AS AMENDED					
(1) FOR	(2) CLAIMS REMAINING AFTER AMENDMENT	(3) HIGHEST NUMBER PAID FOR	(4) NUMBER EXTRA x RATE		(5) ADDITIONAL FEE
TOTAL CLAIMS	36	27	9	x $ 18.00	162.00
INDEPENDENT CLAIMS	5	9	0	x $ 80.00	0.00
MULTIPLE DEPENDENCY FEE				$ 270.00	0.00
			Total Amendment Fee:		$162.00

☒ Please charge Deposit Account No. 01-1300 for: $162.00

The Commissioner is hereby authorized to charge any additional fees required by this paper, including the enclosed documents, and during the entire pendency of this application and to credit any excess amounts paid to Deposit Account No. 01-1300. A copy of this letter is enclosed for use by the Deposit Account Branch.

Respectfully submitted,

Alan M. Gordon
Attorney for Applicants
Reg. No. 30,637

American Home Products Corporation
Patent Law Department
Five Giralda Farms
Madison, NJ 07940-0874
Tel. No. (845) 602-4636

Docket No: 33,383-00
Patent

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re of Application of:	Randall K. Holmes et al.		
Serial No.:	09/806,370	Group Art No.:	To be Assigned
Filed:	September 30, 1999	Examiner:	To be Assigned
For:	Mutant Cholera Holotoxin as an Adjuvant		
Confirmation No.:	8568		
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Commissioner for Patents
Washington, DC 20231

October 3, 2001

Sir:

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10/3/01
Date

Alan M. Gordon
Alan M. Gordon

2. Fee calculation

CLAIMS AS AMENDED					
(1) FOR	(2) CLAIMS REMAINING AFTER AMENDMENT	(3) HIGHEST NUMBER PAID FOR	(4) NUMBER EXTRA x RATE		(5) ADDITIONAL FEE
TOTAL CLAIMS	36	27	9	x $ 18.00	162.00
INDEPENDENT CLAIMS	5	9	0	x $ 80.00	0.00
MULTIPLE DEPENDENCY FEE				$ 270.00	0.00
			Total Amendment Fee:		$162.00

☒ Please charge Deposit Account No. 01-1300 for: $162.00

The Commissioner is hereby authorized to charge any additional fees required by this paper, including the enclosed documents, and during the entire pendency of this application and to credit any excess amounts paid to Deposit Account No. 01-1300. A copy of this letter is enclosed for use by the Deposit Account Branch.

Respectfully submitted,

Alan M. Gordon
Attorney for Applicants
Reg. No. 30,637

American Home Products Corporation
Patent Law Department
Five Giralda Farms
Madison, NJ 07940-0874
Tel. No. (845) 602-4636

33,383-00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Randall K. Holmes et al.

Serial No. : 09/806,370

Filed : September 30, 1999

For : Mutant Cholera Holotoxin as an Adjuvant

Examiner : To be Assigned

Group Art Unit : To be Assigned

Confirmation No.: 8568 Customer No.: 25291

October 3, 2001

Hon. Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

This is a Preliminary Amendment submitted for the above-captioned application. As set forth in the transmittal letter accompanying this Preliminary Amendment, a fee of $162.00 is due for the presentation of additional dependent claims. Please charge $162.00 to Deposit Account

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CERTIFICATION UNDER 37 C.F.R. 1.10

I hereby certify that this paper and the documents referred to as enclosed therein are being deposited with the United States Postal Service on the date written below in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number ET334776318US addressed to the Commissioner for Patents, Washington, D.C. 20231.

10/3/01
Date

Alan M. Gordon
Alan M. Gordon

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No. 01-1300.

Please amend the application as follows:

In the Claims

Please cancel Claims 18-23.

Please amend Claims 1, 24, 26 and 27 as follows:

1. (Amended). An antigenic composition comprising a selected antigen from a pathogenic bacterium, virus, fungus or parasite and an effective adjuvanting amount of a mutant cholera holotoxin, wherein the holotoxin has reduced toxicity compared to a wild-type cholera holotoxin and has a substitution at position 29 of the A subunit of the cholera holotoxin, wherein the glutamic acid residue is replaced by an amino acid other than aspartic acid, and wherein said holotoxin enhances the immune response in a vertebrate host to said antigen.

24. (Amended). A plasmid containing an isolated and purified DNA sequence comprising a DNA sequence which encode an immunogenic mutant cholera holotoxin having a substitution at position 29 of the A subunit of the cholera holotoxin, wherein the glutamic acid residue is replaced by an amino acid other than aspartic acid, and wherein the DNA

sequence is operatively linked to an arabinose inducible promoter.

26. (Amended). A method of producing an immunogenic mutant cholera holotoxin, wherein the cholera holotoxin has reduced toxicity compared to a wild-type cholera holotoxin and has a substitution at position 29 of the A subunit of the cholera holotoxin, wherein the glutamic acid residue is replaced by an amino acid other than aspartic acid, which comprises transforming, transducing or transfecting a host cell with the plasmid of Claim 24 and culturing the host cell under conditions which permit the expression of said recombinant immunogenic detoxified protein by the host cell.

27. (Amended). Use of effective adjuvanting amount of a mutant cholera holotoxin, wherein the holotoxin has reduced toxicity compared to a wild-type cholera holotoxin and has a substitution at position 29 of the A subunit of the cholera holotoxin, wherein the glutamic acid residue is replaced by an amino acid other than aspartic acid, in combination with a selected antigen from a pathogenic bacterium, virus, fungus or parasite, to prepare

an antigenic composition, wherein said holotoxin enhances the immune response in a vertebrate host to said antigen.

Please add Claims 28-42 as follows:

28. (New). The method of Claim 17 wherein the antigenic composition comprises more than one antigen.

29. (New). The method of Claim 17 wherein the amino acid at position 29 is histidine.

30. (New). The method of Claim 17 wherein the selected antigen is the *Haemophilus influenzae* P4 outer membrane protein, the *Haemophilus influenzae* P6 outer membrane protein, the *Haemophilus influenzae* Hap_S protein, the *Helicobacter pylori* urease protein, the *Neisseria meningitidis* rpilin, the *Neisseria meningitidis* PorA protein, the respiratory syncytial virus fusion protein, a rotavirus virus-like particle or HSV gD2.

31. (New). The method of Claim 30 wherein the selected antigen is the *Haemophilus influenzae* P4 outer membrane protein, the *Haemophilus influenzae* P6 outer membrane protein, the *Haemophilus influenzae* Hap_S protein or any combination thereof.

32. (New). The method of Claim 30 wherein the selected antigen is the *Helicobacter pylori* urease protein.

33. (New). The method of Claim 30 wherein the selected antigen is the *Neisseria meningitidis* rpilin, the *Neisseria meningitidis* PorA protein or a combination thereof.

34. (New). The method of Claim 30 wherein the selected antigen is the respiratory syncytial virus fusion protein.

35. (New). The method of Claim 30 wherein the selected antigen is a rotavirus virus-like particle.

36. (New). The method of Claim 35 wherein the virus-like particle is a rotavirus 2/6-virus-like particle.

37. (New). The method of Claim 30 wherein the selected antigen is HSV gD2.

38. (New). The method of Claim 37 wherein the antigenic composition is a polynucleotide vaccine comprising plasmid DNA encoding HSV gD2.

39. (New). The method of Claim 17 wherein the antigenic composition further comprises a diluent or carrier.

40. (New). The method of Claim 17 wherein the antigenic composition further comprises a second adjuvant in addition to the mutant cholera holotoxin.

41. (New). The method of Claim 17 wherein at least one additional mutation is made to the A subunit of the cholera holotoxin at a position other than amino acid 29.

42. (New). The method of Claim 41 wherein the at least one additional mutation is made as a substitution for the arginine at amino acid 7, the aspartic acid at position 9, the arginine at position 11, the histidine at position 44, the valine at position 53, the arginine at position 54, the serine at position 61, the serine at position 63, the histidine at position 70, the valine at position 97, the tyrosine at position 104, the proline at position 106, the histidine at position 107, the serine at position 109, the glutamic acid at position 110, the glutamic acid at position 112, the serine at position 114, the tryptophan at position 127, the arginine at position 146 and the arginine at position 192.

Remarks

Applicants by this Preliminary Amendment are amending Claims 1, 24, 26 and 27 in order to prevent any possible confusion about the meaning of the Claims. As amended, these Claims are directed specifically to a mutation of the glutamic acid residue at amino acid position 29 of the A subunit of the cholera holotoxin. Support for the amendment of these Claims is found at page 4, lines 1-5 of the application. No new matter is added by this amendment.

Applicants are also presenting new dependent Claims 28-42 in place of dependent Claims 18-23. Both sets of Claims depend from Claim 17. Claims 28-42 parallel Claims 2-16, which depend from Claim 1. No new matter is added by this amendment.

Applicants respectfully request that this Preliminary Amendment be entered and that the application be examined on the basis of Claims 1-17 and 24-42.

Respectfully submitted,

Alan M. Gordon
Registration No. 30,637
American Home Products Corp.
Patent Law Department
Five Giralda Farms
Madison, NJ 07940
(845) 602-4636
Attorney for Applicants

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Version With Markings To Show Changes Made

1. (Amended). An antigenic composition comprising a selected antigen from a pathogenic bacterium, virus, fungus or parasite and an effective adjuvanting amount of a mutant cholera holotoxin, wherein the holotoxin has reduced toxicity compared to a wild-type cholera holotoxin and has a substitution [other than aspartic acid for the glutamic acid] at position 29 of the A subunit of the cholera holotoxin, wherein the glutamic acid residue is replaced by an amino acid other than aspartic acid, and wherein said holotoxin enhances the immune response in a vertebrate host to said antigen.

18. (Cancelled).

19. (Cancelled).

20. (Cancelled).

21. (Cancelled).

22. (Cancelled).

23. (Cancelled).

24. (Amended). A plasmid containing an isolated and purified DNA sequence comprising a DNA sequence which encode an immunogenic mutant cholera holotoxin having a

substitution [other than aspartic acid for the glutamic acid] at position 29 of the A subunit of the cholera holotoxin, wherein the glutamic acid residue is replaced by an amino acid other than aspartic acid, and wherein the DNA sequence is operatively linked to an arabinose inducible promoter.

26. (Amended). A method of producing an immunogenic mutant cholera holotoxin, wherein the cholera holotoxin has reduced toxicity compared to a wild-type cholera holotoxin and has a substitution [other than aspartic acid for the glutamic acid] at position 29 of the A subunit of the cholera holotoxin, wherein the glutamic acid residue is replaced by an amino acid other than aspartic acid, which comprises transforming, transducing or transfecting a host cell with the plasmid of Claim 24 and culturing the host cell under conditions which permit the expression of said recombinant immunogenic detoxified protein by the host cell.

27. (Amended). Use of effective adjuvanting amount of a mutant cholera holotoxin, wherein the holotoxin has reduced toxicity compared to a wild-type cholera holotoxin and has a substitution [other than aspartic acid

for the glutamic acid] at position 29 of the A subunit of the cholera holotoxin, wherein the glutamic acid residue is replaced by an amino acid other than aspartic acid, in combination with a selected antigen from a pathogenic bacterium, virus, fungus or parasite, to prepare an antigenic composition, wherein said holotoxin enhances the immune response in a vertebrate host to said antigen.

MUTANT CHOLERA HOLOTOXIN AS AN ADJUVANT

Field of the Invention

This invention relates to the use of an immunogenic mutant cholera holotoxin having reduced toxicity compared to a wild-type cholera toxin and a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the cholera holotoxin as an adjuvant to enhance the immune response in a vertebrate host to a selected antigen.

Background of the Invention

The immune system uses a variety of mechanisms for attacking pathogens. However, not all of these mechanisms are necessarily activated after immunization. Protective immunity induced by immunization is dependent on the capacity of the vaccine to elicit the appropriate immune response to resist or eliminate the pathogen. Depending on the pathogen, this may require a cell-mediated and/or humoral immune response.

A substance that enhances the immune response when administered together with an immunogen or antigen is known as an adjuvant.

The Gram-negative bacterium *Vibrio cholerae* (*V. cholerae*) is the causative agent of the gastrointestinal disease cholera. The diarrhea caused by *V. cholerae* is due to the secretion of cholera toxin (CT).

CT comprises a single A subunit (CT-A), which is responsible for the enzymatic activity of the toxin, and five identical B subunits (CT-B), which are involved in the binding of the toxin to intestinal

epithelial cells, as well as other cells which contain ganglioside GM_1 on their surface. Together, the CT-A and CT-B subunits comprise a holotoxin. The sequence of CT has been described (Bibliography entry 1).

CT a is hexaheteromeric complex consisting of one A polypeptide and five identical B polypeptides (2). The B pentamer is required for binding to the cell surface receptor ganglioside GM_1 (3). The A subunit can be proteolytically cleaved within the single disulfide-linked loop between C187 and C199 to produce the enzymatically active A1 polypeptide (4) and the smaller polypeptide A2, which links fragment A1 to the B pentamer (5). Upon entry into enterocytes, CT-A1 ADP-ribosylates a regulatory G-protein (Gsα), which leads to constitutive activation of adenylate cyclase, increased intracellular concentration of cAMP, and secretion of fluid and electrolytes into the lumen of the small intestine (6). *In vitro*, ADP-ribosyl transferase activity of CT is stimulated by the presence of accessory proteins called ARFs (7), small GTP-binding proteins known to be involved in vesicle trafficking within the eukaryotic cell.

The need for effective immunization procedures is particularly acute with respect to infectious organisms which cause acute infections at, or gain entrance to the body through, the gastrointestinal, pulmonary, nasopharyngeal or genitourinary surfaces. These areas are bathed in mucus, which contains immunoglobulins consisting largely of secretory IgA (8,9,10). This antibody is derived from large numbers of IgA-producing plasma cells which infiltrate the lamina propria regions underlying these mucosal membranes (11,12). IgA is specifically transported to the lumenal surface through the action of the secretory component (13).

However, parenteral immunization regimens are usually ineffective in inducing secretory IgA responses. Secretory immunity is most often achieved through the direct immunization of mucosally-associated lymphoid tissues. Following their induction at one mucosal site, the precursors of IgA-producing plasma cells extravasate and disseminate to diverse mucosal tissues where final differentiation to high-rate IgA synthesis occurs (14,15,16). Extensive studies have demonstrated the feasibility of mucosal immunization to induce this common mucosal immune system (17), but with rare exceptions the large doses of antigen required to achieve effective immunization have made this approach impractical for purified vaccine antigens. Among the strategies investigated to overcome this problem is the use of mucosal adjuvants. It is known that CT is one of the most potent adjuvants, and that the co-administration of CT with an unrelated antigen results in the induction of concurrent circulating and mucosal antibody responses to that antigen (18). Thus, CT can act as an adjuvant.

It would be preferable to use as an adjuvant a form of the CT holotoxin that has reduced toxicity so as to reduce the undesirable symptoms of diarrhea caused by wild-type CT. Thus, there is a need to identify a mutant CT holotoxin which is able to enhance the immune response while reducing the toxicity of the CT holotoxin.

Summary of the Invention

Accordingly, it is an object of this invention to utilize a mutant form of the CT holotoxin that has reduced toxicity compared to a wild-type CT as an adjuvant in an antigenic composition to enhance the

immune response in a vertebrate host to a selected antigen from a pathogenic bacterium, virus, fungus or parasite.

These objects of the invention are achieved with a mutant cholera holotoxin featuring a point mutation at amino acid 29 of the A subunit, wherein the glutamic acid residue is replaced by an amino acid other than aspartic acid. In a particular embodiment of this invention, the amino acid 29 is histidine. The mutated CT (also referred to as CT-CRM) is useful as an adjuvant in an antigenic composition to enhance the immune response in a vertebrate host to a selected antigen from a pathogenic bacterium, virus, fungus or parasite. The mutant CT is produced by site-directed mutagenesis of the DNA encoding the wild-type CT using conventional techniques. The antigenic composition may further comprise a diluent or carrier.

The invention is also directed to methods for increasing the ability of an antigenic composition containing a selected antigen from a pathogenic bacterium, virus, fungus or parasite to elicit the immune response of a vertebrate host by including an effective adjuvanting amount of a mutant cholera holotoxin, wherein the holotoxin has reduced toxicity compared to a wild-type CT and the glutamic acid at amino acid position 29 of the A subunit of the cholera holotoxin is replaced by an amino acid other than aspartic acid, in particular a histidine.

The invention further relates to plasmids containing isolated and purified DNA sequences comprising DNA sequences which encode an immunogenic mutant cholera holotoxin having a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the cholera holotoxin, and wherein such a DNA sequence is operatively linked to an

arabinose inducible promoter, as well as to suitable host cells transformed, transduced or transfected with such plasmids. The immunogenic mutant cholera holotoxin is produced by transforming, transducing or transfecting a host cell with a plasmid described above and culturing the host cell under conditions which permit the expression of said recombinant immunogenic detoxified protein by the host cell.

Brief Description of the Figures

Figure 1 depicts the capacity of CT-CRMs to bind ganglioside GM_1. Each CT-CRM was diluted three-fold at an initial concentration of 3 μg/ml and tested in duplicate. The binding capacity was expressed as the mean absorbance at 410 nm for each dilution.

Figure 2 depicts the reduction in nasal colonization in mean Log_{10} cfu per nose in mice immunized intranasally (n = 10 per group) with meningococcal recombinant pilin (rpilin) with or without CT-CRM_{E29H} adjuvant, or non-immunized mice, where each group was then challenged with the homologous meningococcal bacterial strain.

Figure 3 depicts the reduction in nasal colonization in mean Log_{10} cfu per nose in mice immunized intranasally (n = 5 per group) with meningococcal rpilin with or without CT-CRM_{E29H} adjuvant, meningococcal class 1 outer membrane protein (Por A) with CT-CRM_{E29H} adjuvant, KLH with CT-CRM_{E29H} adjuvant, or non-immunized mice, where each group was then challenged with the homologous meningococcal bacterial strain.

Figure 4 depicts the reduction in nasal colonization in mean Log_{10} cfu of meningococcal strain

870227 per nose in mice immunized intranasally (n = 10 per group) with meningococcal rpilin, PorA from meningococcal strain H355, PorA from meningococcal strain 870227, heat-inactivated meningococcal strain 870227 whole cells or KLH, each adjuvanted with CT-CRM_{E29H}, where each group was then challenged with a heterologous meningococcal bacterial strain (870227).

Figure 5 depicts the reduction in nasal colonization in mean Log_{10} cfu of meningococcal strain 870227 per nose in mice immunized subcutaneously (n = 10 per group) with PorA from meningococcal strain H355 with CT-CRM_{E29H} or MPL™ adjuvant, KLH with MPL™ adjuvant, or heat-inactivated meningococcal strain 870227 whole cells with MPL™ adjuvant, where each group was then challenged with a heterologous meningococcal bacterial strain (870227).

Figure 6 depicts a first assay of the antigen-dependent cytolytic activity to respiratory syncytial virus (RSV)-infected target cells as the percentage of cell lysis versus effector:target ratio.

Figure 7 depicts a first assay of the protection of mouse lung to RSV challenge by immunization with F protein plus adjuvant, where the lung virus titer is measured as Log_{10} PFU per gram.

Figure 8 depicts a second assay of the antigen-dependent cytolytic activity to RSV-infected target cells as the percentage of cell lysis versus effector:target ratio.

Figure 9 depicts a second assay of the protection of mouse lung to RSV challenge by immunization with F protein plus adjuvant, where the lung virus titer is measured as Log_{10} PFU per gram.

Figure 10 depicts rotavirus-specific serum antibody responses in BALB/c mice immunized intranasally with 2/6-VLPs with or without CT-CRM_{E29H}

adjuvant. BALB/c mice were immunized intranasally with 2/6-VLPs with (n=4) or without (n=5) CT-CRM_{E29H} and levels of rotavirus-specific IgG (Figure 10A), IgM (Figure 10B) and IgA (Figure 10C) were measured. Standard deviations are shown.

Figure 11 depicts rotavirus-specific serum antibody responses in BALB/c inbred mice immunized with 2/6-VLPs. Groups of BALB/c mice were immunized, orally (square, n=4), intranasally (diamond, n=5) or in combination (intranasal plus oral, circle, n=4), with 2/6-VLPs plus CT-CRM_{E29H} on week 0 and 2. Serum samples were collected from individual mice in each group on weeks shown, and levels of serum IgG (Figure 11A), IgM (Figure 11B) and IgA (Figure 11C) were determined for each mouse by ELISA. Geometric mean titers (GMT) were calculated for each group and plotted against weeks post-immunization.

Figure 12 depicts IgG1 and IgG2a antibody subclasses in BALB/c mice. Pre-challenge sera of BALB/c mice immunized orally or IN, with rotavirus 2/6-virus-like particles (VLPs) plus CT-CRM_{E29H}, were used to determine IgG subclasses. Standard deviations are shown.

Figure 13 depicts rotavirus-specific intestinal antibody responses in inbred BALB/c mice immunized with 2/6-VLPs. Groups of BALB/c mice were immunized with 2/6-VLPs plus CT-CRM_{E29H} as described for Figure 11, and levels of rotavirus-specific intestinal IgA (Figure 13A) and IgG (Figure 13B) were measured. No rotavirus-specific intestinal IgM was detected in any mice.

Figure 14 depicts protection of 2/6-VLP immunized BALB/c and CD-1 mice following challenge with murine rotavirus. Figure 14A depicts a comparison of

percent reduction in antigen shedding (PRAS) between mice immunized with 2/6-VLPs with (n=5) or without (n=4) CT-CRM_{E29H}. PRAS for each mouse (•) and the mean of each group (–) were calculated. Figure 14B depicts groups of BALB/c mice immunized with 2/6-VLP plus CT-CRM_{E29H} by different routes as shown, and protection levels were determined as described above. Figure 14C depicts groups of outbred CD-1 mice immunized with 2/6-VLPs plus CT-CRM_{E29H} orally and intranasally as described above. On week 26, immunized and control mice were challenged and PRAS calculated as described above. In the oral group (n=4), one mouse died before challenge (n=3 for protection).

Detailed Description of the Invention

The utility of mutant forms of CT as adjuvants for antigenic compositions is described herein. A set of mutant CT clones (CT-CRMs) in *E. coli* was generated. The data indicate that the CT-CRM with superior adjuvanting properties is the mutant with a nonconservative amino acid substitution (glutamic acid to histidine) at position 29 in the A subunit (CT-CRM_{E29H}). The cumulative data demonstrate that CT-CRM_{E29H} is a holotoxin and is less toxic than wild-type CT. Importantly, CT-CRM_{E29H} is able to augment mucosal and systemic immune responses following either intragastric (IG) or intranasal (IN) administration of disparate vaccine antigens. These vaccine antigens are from either bacterial or viral pathogens. Results in the murine models of *Helicobacter felis*, rotavirus and respiratory syncytial virus (RSV) infection indicate that the immune responses facilitated by intragastric or intranasal immunization with a CT-CRM_{E29H}-prepared

vaccine are protective. The data indicate that CT-CRM_{E29H} is at least as active as an adjuvant as wild-type CT. Even in the presence of pre-existing anti-CT immune responses, CT-CRM_{E29H} is able to serve as a mucosal adjuvant.

The mutant CT-A retained its ability to assemble with CT-B to form a holotoxin that resembled wild-type CT in its adjuvanticity, but exhibited reduced toxicity compared to a wild-type CT. The B subunits may have their native sequence or may themselves be mutated.

The resulting reduced level of toxicity provides an altered CT for use as an adjuvant. The immunogenic mutant CT according to the present invention exhibits a balance of reduced toxicity and retained adjuvanticity, such that the protein functions as an adjuvant while being tolerated safely by the vertebrate host immunized with the antigenic composition.

The antigenic compositions of the present invention modulate the immune response by improving the vertebrate host's antibody response and cell-mediated immunity after administration of an antigenic composition comprising a selected antigen from a pathogenic bacterium, virus, fungus or parasite and an effective adjuvanting amount of a mutant CT, where the CT has reduced toxicity compared to a wild-type CT and the glutamic acid at position 29 of the A subunit of the cholera holotoxin is replaced by an amino acid other than aspartic acid. In a particular embodiment of this invention, the amino acid 29 is histidine.

As used herein, the term "the holotoxin has reduced toxicity" means that the CT-CRM mutant, such as the CT-CRM_{E29H} mutant, exhibits a substantially lower

toxicity per unit of purified toxin protein compared to the wild-type CT, which enables the mutant to be used as an adjuvant in an antigenic composition without causing significant side effects.

As used herein, the term "effective adjuvanting amount" means a dose of the CT-CRM mutant, such as the CT-CRM_{E29H} mutant, which is suitable to elicit an increased immune response in a vertebrate host. The particular dosage will depend upon the age, weight and medical condition of the host, as well as on the method of administration. Suitable doses are readily determined by persons skilled in the art.

Five CT-CRMs were generated as described in Example 1 below with the following mutations in the A subunit:

Amino Acid	Native	Mutant	Abbreviation
7	arginine	lysine	CT-CRM_{R7K}
11	arginine	lysine	CT-CRM_{R11K}
29	glutamic acid	histidine	CT-CRM_{E29H}
110	glutamic acid	aspartic acid	CT-CRM_{E110D}
112	glutamic acid	aspartic acid	CT-CRM_{E112D}

The phenotypic effects of these mutations on structure and function of CT were then assessed.

The variant CT-A's R7K, E29H, E110D and E112D were able to assemble into immunoreactive holotoxin as determined by a ganglioside GM_1 binding assay (Figure 1). However, a portion of purified R11K did not appear to be a holotoxin when tested with the polyclonal antibodies described in Example 2.

Each holotoxin variant was tested in a Y-1 adrenal tumor cell assay (19) to determine its residual toxicity compared to wild-type CT holotoxin. The

results presented in Table 2 demonstrated that CT-CRM_{E29H} and commercial CT-B (Sigma) had 1.2% residual toxicity. The 1.2% residual toxicity associated with commercial CT-B was most likely due to contaminating A subunit (approximately 0.5%). The residual toxicity of the remaining CT-CRMs with mutations at amino acid positions 7, 11, 110, or 112 were less than or equal to 0.4%.

CT-CRM_{E29H} was tested in the patent mouse gut weight assay (20) to estimate intestinal fluid accumulation as an *in vivo* measure of toxicity. The results presented in Table 3 demonstrated that CT-CRM_{E29H} was significantly less active in stimulating an increase in fluid accumulation into the intestinal tract of mice than was wild-type CT.

Each CT-CRM was also compared to CT in an ADP-ribosyltransferase activity assay. The results were generally in agreement with those generated in the Y-1 adrenal cell assay and suggested that mutation in the A1 subunit resulted in diminished ADP-ribosyltransferase activity by the various CT-CRMs when compared to wild-type CT (Table 4). The mutant with the largest enzyme activity appeared to be CT-CRM_{E29H}. This activity was approximately 10% that of wild-type CT.

Trypsinization at 37°C of CT-CRM_{E29H} caused cleavage of CT-A into fragments A1 and A2 in a manner indistinguishable from treatment of wild-type CT based on Western blot analyses. This provides further evidence that the structure of CT-CRM_{E29H} is similar to that of wild-type CT.

The apparent differences in activity of CT-CRM_{E29H} in the Y-1 adrenal tumor cell and ADP-ribosylation activity assays are due to trypsin

activation of the mutant holotoxin in the latter assay. Thus, the lack of CT-A cleavage into A1 and A2 subunits due to the reduced protease activity in *E. coli* contributes to the attenuation of the *E. coli*-expressed CT-CRM_{E29H}. Collectively, the accumulated data show that CT-CRM_{E29H} is a holotoxin that binds to ganglioside GM_1 and is significantly less toxic than wild-type CT.

A series of studies was conducted to evaluate the efficacy of CT-CRM_{E29H} as a mucosal adjuvant for compositions containing bacterial or viral antigens which have been identified as vaccine candidates as follows: (1) Nontypable *Haemophilus influenzae* (NTHi) recombinant P4 protein, also known as protein "e" (rP4) (21), recombinant NTHi P6 protein (rP6) (22), and purified native *Haemophilus influenzae* adherence and penetration (Hap_S) protein (23); (2) *Helicobacter pylori* recombinant Urease protein (rUrease) (24); (3) *Neisseria meningitidis* Group B recombinant class 1 pilin (25) and *Neisseria meningitidis* Group B class 1 outer membrane protein (26); (4) Respiratory syncytial virus purified native fusion protein (RSV F) (27); and (5) 2/6-virus-like particles of rotavirus (28).

CT-CRM_{E29H} was compared to four other CT mutants and wild-type CT as an adjuvant for the NTHi rP4 and rP6 proteins. The results indicated that the five different CT-CRMs augmented the capacity of rP4 and rP6 proteins to elicit systemic humoral immune responses (Tables 5 and 6). For example, two weeks after tertiary IN immunization the anti-rP4 IgG antibody titers of mice immunized with rP4 and rP6 proteins formulated with either CT-CRM_{E29H} or CT-CRM_{E110D} were 40 times greater that of mice immunized with the recombinant proteins in PBS alone (Table 5). The antibody titers of mice administered the recombinant

proteins plus wild-type CT holotoxin were elevated 20-fold. The anti-rP4 antibody titers of mice immunized with the CT-CRM_{R11K} were elevated 10-fold.

Even more dramatic differences were observed when the sera were examined for anti-native P6 antibody titers (Table 6). Two weeks after secondary IN immunization the serum anti-native P6 antibody titers of mice immunized with either the CT-CRM_{E29H} or CT-CRM_{E110D} formulated vaccines were more than 30 times greater than that of mice immunized with rP6 plus PBS. In comparison, the vaccine prepared with wild-type CT elicited anti-native P6 antibody titers that were 90 times greater than that generated by the PBS prepared formulation. The anti-native P6 antibody titers of mice immunized with either the CT-CRM_{E112D}, CT-CRM_{R7K}, or CT-CRM_{R11K} preparations were only two to four times greater than that of recipients immunized with rP4 plus rP6 formulated with PBS alone.

An examination of the protein-specific antibodies in the mucosal secretions two weeks after tertiary immunization further indicated that the CT-CRMs facilitated the generation of local immune responses against the rP4 protein. Moreover, the anti-rP4 antibody titers were comparable to those induced by wild-type CT (Table 7). Local antibody titers were not detected against native P6 protein (data not shown). Thus, the data when taken together suggested that the most propitious mutant CTs for generating both systemic and local antibody responses against rP4 and rP6 proteins were the CT-CRMs which contained a mutation at either position 29 or 110.

An additional study was performed to confirm the potential of CT-CRM_{E29H} as an adjuvant and determine the appropriate dose for IN immunization (Table 8).

The results indicated that 1 μg of CT-CRM_{E29H} facilitated the greatest systemic and local humoral immune responses against rP4 protein. When the dose of CT-CRM_{E29H} was increased from 1 to 10 or 30 μg per dose, the data suggested that both systemic and mucosal immune responses were diminished. For example, the serum anti-P4 IgG antibody titers of mice immunized with 10 μg CT-CRM_{E29H} was one-seventh that of mice immunized with the 1 μg CT-CRM_{E29H} on day 48 of the study (Table 8). Moreover, the local anti-P4 IgA antibody titers from the bronchoalveolar and vaginal wash fluids of the former group were one-thirty-fourth and one-sixteenth that of the latter group of mice on day 49. The data indicated that the local and systemic humoral immune responses of mice immunized with rP4 plus rP6/CT-CRM_{E29H} were essentially identical to those attained after immunization with the wild-type CT adjuvanted vaccine (Table 8).

The effect of the addition of CT-CRM_{E29H} on the serum antibody responses elicited by immunization with the Hap_S protein was examined. Addition of CT-CRM_{E29H} helped induce a serum antibody response to the Hap_S protein (Table 9). The immune response was seen in week 7 sera; no antibody titers were detected in earlier sera. The anti-Hap_S ELISA titers of the sera obtained from immunized mice are shown in Table 9. The responses increased in a dose dependent manner and were augmented approximately three-fold by adddition of 0.1 μg of CT-CRM_{E29H}. This augmentation occurred at both dosage levels.

The potential of the five different CT-CRMs to augment systemic and local humoral immune responses after intragastric (IG) immunization with the rUrease

protein of *H. pylori* was assessed using a mouse model (29). The results were similar to those obtained with the NTHi proteins after intranasal administration. The data indicated that CT-CRM_{E29H} was the mutant with the most potential for augmenting systemic and local humoral immune responses after IG immunization. The geometric mean serum anti-rUrease IgG (Table 10) and IgA (Table 11) antibody titers elicited by the CT-CRM_{E29H} formulated vaccine were six and three times greater, respectively, than those induced by CT-CRM_{E110D} on day 28 of the study. Furthermore, the serum IgG and IgA antibody titers elicited by the CT-CRM_{E29H} formulated vaccine were equivalent to that generated by the vaccine containing wild-type CT.

Most importantly, IG immunization with the rUrease formulated with CT-CRM_{E29H} appeared to generate the greatest local humoral immune responses (Table 12). This was most evident after the examination of the bronchoalveolar wash fluids. The anti-rUrease IgA antibody titers in the bronchoalveolar wash fluids were five times greater than that elicited by the CT-CRM_{E110D} prepared vaccine. In comparison to the wild-type CT formulation, the anti-rUrease IgA antibody titers were at one-fifth the level. However, the protein-specific IgA antibody titers in the vaginal wash fluids of the group immunized with the CT-CRM_{E29H} formulated vaccine were essentially equivalent to those elicited by the wild-type CT prepared vaccine (Table 12).

It was noteworthy that the data imply that parenteral immunization did not elicit remarkable rUrease-specific IgA antibodies in the bronchoalveolar wash fluids when compared to those elicited in mice immunized IG with the CT-CRM_{E29H} prepared vaccine (Table 12).

Therefore, a second study was conducted to test the efficacy of the immune responses generated by rUrease formulated with CT-CRM_{E29H}. The data suggest that CT-CRM_{E29H} is as potent as wild-type CT in supporting the induction of protective immune responses against *H. felis* (Table 13). The serum anti-rUrease IgA antibody titers of the former group were equivalent to those of the latter group of mice on day 28 of the study. The protein-specific IgA antibody titers in the sera of mice parenterally immunized with rUrease plus Stimulon™ QS-21 were 12 times greater than those of mice immunized IG with the CT-CRM_{E29H} prepared vaccine. However, the protein-specific IgA antibody titers in the bronchoalveolar wash fluids of mice immunized with CT-CRM_{E29H} were more than ten times greater than those of parenterally immunized mice (Table 13).

The results suggested a correlation between IG immunization and the ability of mice to clear *H. felis* from the stomach tissue. Ten days after the last challenge, 80% of the of mice immunized IG with vaccines formulated with either CT or CT-CRM_{E29H} were able to clear urease-containing bacteria from the stomach tissues. In contrast, naive control mice (10%), mice immunized IG with rUrease plus PBS alone (20%), or mice immunized subcutaneously with rUrease admixed with Stimulon™ QS-21 (30%) appeared to have less ability to eradicate *H. felis* (Table 13). It was noteworthy that the data did not suggest a relationship between efficacy and protein-specific IgA antibody titers in the bronchoalveolar wash fluids. The protein-specific IgA antibody titers in the bronchoalveolar wash fluids of mice immunized IG with rUrease plus wild-type CT were one-tenth those of mice immunized with CT-CRM_{E29H} (Table 13). Yet 80%

protection was achieved with either vaccine. Thus, monitoring local humoral immune responses in the pulmonary tissues may have little relevance to protective immune responses that occur in the stomach.

It has been suggested by Dr. Jani O'Rourke (University of New South Wales; personal communication) that C57Bl/6 mice, unlike BALB/c mice, experience disease similar to that observed in humans after infection with *H. pylori*. To test the efficacy of the anti-rUrease immune responses facilitated by CT-CRM_{E29H} to clear *H. pylori* from the gastric tissues, a separate series of studies were initiated using C57Bl/6 mice. The results suggested that IG immunization with rUrease formulated with CT-CRM_{E29H} generated systemic and local humoral immune responses that were similar to those elicited by rUrease formulated with wild-type CT (Table 14). The serum and bronchoalveolar and vaginal wash fluid anti-rUrease IgA antibody titers of mice immunized with either wild-type CT or CT-CRM_{E29H} prepared vaccines on day 28 of the study were indistinguishable. The only disparities were the IgA antibody titers detected in extracts of the fecal pellets from the mice immunized with the CT-CRM_{E29H} prepared vaccine (Table 14), which were three times greater. It was noteworthy that the protein-specific IgA antibody titers in the feces of mice parenterally immunized with rUrease plus alum were substantially lower than those of mice IG immunized with either wild-type CT or CT-CRM_{E29H} formulations (one thirty-eighth and one-fourteenth, respectively). Thus, the C57Bl/6 mouse model appeared capable of assessing the capacity of CT-CRM_{E29H} to adjuvant immune responses generated after IG immunization. Moreover, the data indicated that the model was capable of defining the roles of

local and systemic immune responses in protecting mucosal surfaces from *H. pylori*.

It has been reported that CT is contraindicated as an adjuvant for mucosal immune responses (30,31). The hypothesis was that CT predisposed vaccines to elicit heightened IgE antibody titers, which are undesirable. IgE is associated with hypersensitivity and allergic reactions. It was further implied that the heat-labile toxin of *E. coli* (LT), or LT-CRMs, had less potential to elicit heightened IgE antibody titers. Hence, the conclusion was that LT or LT-CRMs are more appropriate vaccine adjuvants for the generation of mucosal immune responses. To test this hypothesis, vaccines composed of rUrease were formulated with either CT, LT, or CT-CRM_{E29H} and tested in C57Bl/6 mice for their ability to elicit IgE antibodies after IG immunization (Table 15). The data suggested that vaccines prepared with either CT-CRM_{E29H} or wild-type CT were less likely than wild-type LT to generate total or urease-specific IgE antibodies in the circulation. Indeed, the implication was that vaccines formulated with CT-CRM_{E29H} were less likely to generate elevated IgE antibody titers. Both the endpoint total and the rUrease-specific IgE antibody titers were one-fourth those of mice immunized with the vaccine prepared with wild-type LT (Table 15). Thus, these data suggest that, at least in a rUrease formulation, CT-CRM_{E29H} is preferred over LT as an adjuvant.

Without being bound by theory, the mechanism of LT activity recently proposed by van den Akker et al. (32) presents a more likely explanation for the reduced toxicity of CT variants altered in or around E29. After cleavage of the disulfide loop and

reduction of the disulfide bond linking domains A1 and A2, the loop consisting of residues 30-33 in the A1 domain is proposed to change position as a consequence of movement of the long helix of domain A2. Substitutions for E29 may alter the behavior of loop 30-33, resulting in decreased activation of CT-A1. A potential explanation for the reduced toxicity of CT-Y30WAH and CT-G34GGP may also be explained by effects on the 30-33 loop that are detrimental to activation of CT-A1. The next step in the proposed activation pathway is movement of the entire 25-36 loop, which disrupts interactions between R25 and Y55. CT-R25G showed a reduction in toxicity to a greater extent than CT-R25W, possibly because the side chains of R25 and Y55 participate in hydrophobic interactions, which may be retained by CT-R25W but not CT-R25G. The phenotypes of our variants are consistent with the model for activation of heat-labile enterotoxins proposed by van den Akker et al. (32).

A series of experiments was conducted to evaluate the efficacy of CT-CRM_{E29H} as a mucosal and parenteral adjuvant for two vaccine candidates from *Neisseria meningitidis* Group B. The first candidate was a recombinant class 1 pilin (rpilin) (25). The second candidate was a class 1 outer membrane protein (PorA) expressed by a mutant meningococcal strain that did not express the class 2/3 protein (26).

A mucosal adjuvant effect was shown in a first experiment, in that rpilin-specific serum IgG antibodies were enhanced in CT-CRM_{E29H} added groups, ranging from 3 to 19-fold increases in comparison to the titers obtained in mice receiving rpilin in saline (Table 16). Specific serum IgA also increased 2 to 5-fold in the mice immunized with rpilin delivered in CT-CRM_{E29H} (at both 0.1 and 1.0 μg). It is noteworthy that

CT-CRM_{E29H} (1 μg) increased rpilin-specific IgA 3 to 10-fold in the nasal, vaginal, and bronchoalveolar washes. Furthermore, IN immunization with rpilin plus CT-CRM_{E29H} significantly reduced nasal colonization by the Group B *N. meningitidis* homologous strain to the level of detection in Swiss-Webster mice (Figure 2).

A second experiment was conducted to demonstrate that CT-CRM_{E29H} enhanced protection of rpilin against a homologous meningococcal strain. As shown in Table 17, sera IgG titers of meningococcal B whole cell ELISA were increased in the rpilin plus CT-CRM_{E29H} group at least four-fold compared to the homologous strain, as well as to heterologous strains FAM18 and M982 in comparison to the titers from mice receiving rpilin alone. As a control, mice immunized with KLH plus CT-CRM_{E29H} did not induce sera IgG in whole cell ELISA to any of the strains tested. In Table 18, rpilin-specific IgG and IgA antibodies were substantially increased in the rpilin plus CT-CRM_{E29H} group, as compared to the unadjuvanted group. Moreover, CT-CRM_{E29H} as a mucosal adjuvant for rpilin protected mice against nasal colonization by the homologous Group B meningococcal strain (Figure 3).

Next, the immunogenicity of PorA plus CT-CRM_{E29H} in IN immunization was demonstrated. The group receiving PorA adjuvanted with CT-CRM_{E29H} generated increased serum IgG antibodies to *N. meningitidis* H44/76 whole cells and generated 7 and 14-fold higher PorA-specific antibodies compared to the unadjuvanted PorA group (Table 19). However, serum IgA antibodies to PorA H44/76 were not detectable. There were also no PorA-specific antibodies detected in any of the mucosal secretion samples collected (Table 19).

A fourth experiment was conducted to demonstrate that CT-CRM_{E29H} enhanced protection by either rpilin or PorA against a heterologous meningococcal strain. The data, particularly from Table 20, indicate that IN administration of class 1 rpilin or PorA delivered with CT-CRM_{E29H} not only elicited high serum antibody titers to antigens and meningococcal whole cells, but also protected Swiss-Webster mice against nasal colonization by a heterologous strain of group B *N. meningitidis*. Specifically, CT-CRM_{E29H} enhanced serum antibody response to class 1 rpilin as compared to that of the unadjuvanted group. Similarly, the adjuvanted class 1 rpilin group provided enhanced nasal clearance of the bacteria.

Next, the ability of CT-CRM_{E29H} to serve as an adjuvant for meningococcal parenteral immunization was examined. As shown in Figure 5, PorA H355 adjuvanted with either MPL™ or CT-CRM_{E29H} significantly reduced nasal colonization of Group B meningococcal heterologous strain 870227. In particular, mice immunized subcutaneously with PorA H355 plus CT-CRM_{E29H} had even significantly fewer colonies than the PorA H355 plus MPL™ group in the nose 24 hours post-challenge. However, bactericidal activities were detected in the sera only from the PorA and the heat killed whole cell adjuvanted with MPL™ immunized groups respectively (Table 22), but not from the PorA plus CT-CRM_{E29H} group. Even though PorA adjuvanted with CT-CRM_{E29H} did not elicit homologous bactericidal activities similar to that of MPL™ adjuvant, it was highly efficacious in reducing the colonization by a heterologous Group B meningococcal strain.

The capacity of CT-CRM_{E29H} to augment systemic and mucosal immune responses against respiratory syncytial virus (RSV) glycoproteins was examined using the purified native fusion (F) protein. In addition, the impact of pre-existing anti-CT antibodies on the potency of CT-CRM_{E29H} as an adjuvant was investigated. The results demonstrated that BALB/c mice immunized IN with F protein adjuvanted with either CT or CT-CRM_{E29H} generated systemic and local anti-CT IgG and IgA antibody titers (Table 23). Moreover, the data indicated that the antibody titers generated by the formulation containing CT-CRM_{E29H} were equivalent to those elicited by the formulation containing wild-type CT. For example, 10 days after secondary immunization with F protein/CT-CRM_{E29H} (1 μg per dose), the serum anti-CT IgA and IgG antibody titers were only slightly lower than those of mice immunized with F protein/CT (1 μg per dose). Similar results were also obtained after examination of the vaginal wash fluids from mice immunized with F protein prepared with either 1 or 10 μg CT-CRM_{E29H} (Table 23). The data therefore suggested that CT-CRM_{E29H} was as immunogenic as wild-type CT.

The question of whether anti-CT immune responses could adversely affect the immunogenicity of the F antigen was addressed in a second experiment where BALB/c mice were primed first by two IN administrations with either wild-type CT or CT-CRM_{E29H} in PBS alone (Table 24). Thereafter, the appropriate mice were immunized twice with F protein admixed with either wild-type CT or CT-CRM_{E29H}. An examination of the sera collected two weeks after the last administration (day 56) indicated that pre-existing anti-CT antibodies did not have a negative impact on

the level of local or systemic anti-F protein IgA and IgG antibodies. Indeed, the data indicated that pre-existing anti-CT antibodies were beneficial for the generation of an augmented anti-F protein antibody response. This was most evident when the anti-F protein antibody titers elicited at mucosal surfaces were compared (Table 24). Two weeks after secondary immunization, the anti-F protein IgA antibody titers in the bronchoalveolar and vaginal wash fluids of mice primed first with CT-CRM_{E29H} and then immunized with F protein/CT-CRM_{E29H} were 7 and 17 times greater, respectively, than that of naive mice immunized solely with F protein/CT-CRM_{E29H} (Table 24).

In a third experiment, systemic and mucosal immune responses of BALB/c mice immunized IN with RSV F protein and CT-CRM_{E29H}, CT-B or alum were assessed. Table 25 sets forth the humoral immune responses of sera collected nine days post-tertiary immunization. Mice that had received immunizations containing F protein and either 1 or 10 μg of CT-CRM_{E29H} (groups 777 and 778, respectively) displayed significantly elevated titers for IgG, IgG1 and IgG2a when compared to mice immunized with F/PBS, F/AlOH or RSV (groups 784, 785 and 907, respectively). In addition, the titers generated by vaccines containing F/CT-CRM_{E29H} (777 and 778) were at least equivalent to those stimulated by F protein and CTB (779 and 780).

Bronchoalveolar lavage fluids, vaginal and nasal washes were collected from the immunized animals one week post-final immunization in order to perform IgG and IgA antibody ELISAs. The data, set forth in Table 26, show titers from pools of five mice. Mice immunized with CT-CRM_{E29H} elicited detectable IgA in both vaginal and nasal washes (groups 777 and 778).

IgA was not seen in BALs derived from mice immunized with CT-CRM_{E29H} and this represented a contrast to that seen in the BAL of RSV immunized mice (group 907) and F/CTB immunized mice (780). IgG was seen in all mucosal washes including that from the BAL. The levels of IgG seen in the washes from CT-CRM_{E29H} immunized mice were comparable to those obtained by immunizing with CTB (groups 779 and 780) and live RSV.

In a fourth experiment, the cytolytic (CTL) activity elicited by *in vitro* stimulated spleen cells derived from immunized mice was assessed. The data are presented in Figure 6. Whereas RSV-immunized mice showed antigen-specific cell lysis of approximately 60%, the CTL activity of each of the remaining mice remained less than 20%. Thus, whereas CT-CRM_{E29H} was able to induce both systemic and mucosal humoral immune responses to RSV F protein (Tables 25 and 26), cell-mediated immune responses to RSV-infected target cells were not observed.

In a fifth experiment, viral protection assays were performed in order to investigate whether the intranasal delivery of F/CT-CRM_{E29H} facilitates protection against live RSV challenge. The data are presented in Figure 7.

Statistical analysis by ANOVA of the results depicted in Figure 7 is as follows:

$p < 0.05$: F/PBS versus F/CT-CRM_{E29H} (1 μg and 10 μg CT-CRM_{E29H}), F/CTB (1 μg and 10 μg), F/AlOH.

$p > 0.05$: PBS/CT-CRM_{E29H} versus F/PBS

$p > 0.05$: F/CT-CRM_{E29H} (1 μg and 10 μg CT-CRM_{E29H}) versus F/CTB (1 μg and 10 μg) versus F/AlOH.

Mice that received IN vaccines containing F/CT-CRM_{E29H} or F/CTB had lung viral titers comparable to those achieved in mice immunized intramuscularly with F/AlOH ($p>0.05$). Furthermore, intranasal immunization with F/CT-CRM_{E29H} was seen to reduce lung virus titers by Log_{10} 1.6 and Log_{10} 1.4 compared to IN immunization with F/PBS or PBS/ CT-CRM_{E29H}, respectively. The differences between F/CT-CRM_{E29H} and F/PBS or PBS/CT-CRM_{E29H} were found to be statistically significant ($p<0.05$).

In a sixth experiment, systemic and mucosal immune responses of BALB/c mice immunized IN with RSV F protein and CT-CRM_{E29H} or alum were assessed. Table 27 sets forth the humoral immune responses of sera collected two weeks post-tertiary immunization. Mice that received immunizations containing F protein and 1 μg of CT-CRM_{E29H} (group 256) displayed significantly elevated titers for IgG, IgG1 and IgG2a when compared to mice immunized with F/PBS or PBS/CT-CRM_{E29H} (groups 250 and 257, respectively). No significant differences were observed in the IgG1 titers between mice immunized with F/CT-CRM_{E29H} (256) and F/AlOH (258). However, IN immunization with F/CT-CRM_{E29H} (256) elicited significantly elevated IgG2a titers compared to those seen by immunization with F/AlOH (258). Collectively, these results are in agreement with those presented in Table 25. Whereas serum IgA was detected in groups of mice receiving F/CT-CRM_{E29H}, the titers were much lower than previously observed (16,202±2,031 for group 777 and 444±1,458 for group 256). The reasons for the

apparent difference are not clear. Nevertheless, the ability of F/CT-CRM_{E29H} delivered IN to induce serum IgA is consistent in both studies and contrasts favorably with the capacity of F/AlOH in this regard.

Bronchoalveolar lavage fluids, vaginal and nasal washes were collected from the immunized animals two weeks post-final immunization in order to perform IgG and IgA antibody ELISAs. The data, set forth in Table 28, show titers from pools of five mice. Similar to the results shown in Table 26, mice immunized with CT-CRM_{E29H} elicited detectable IgA in both vaginal and nasal washes (group 256). Again similar to the data presented in Table 26, IgA was not seen in BALs derived from mice immunized with CT-CRM_{E29H}. However, IgG was seen in all of the mucosal washes including that from the BAL. The levels of IgG observed in the washes from F/CT-CRM_{E29H}-immunized mice were at least comparable to those obtained by immunizing with live RSV (Table 28, groups 256 versus 259).

In a seventh experiment, the cytolytic (CTL) activity elicited by *in vitro* stimulated spleen cells derived from immunized mice was assessed. The data are presented in Figure 8. Whereas RSV-immunized mice showed antigen-specific cell lysis of approximately 45%, the CTL activity of each of the remaining mice remained less than 10%. The data confirm the inability of IN immunization with F/CT-CRM_{E29H} to induce a cell-mediated immune defense mechanism against RSV-infected target cells in the splenic lymphocyte population. This confirms the previous observation (Figure 8).

In an eighth experiment, additional viral protection assays were performed in order to investigate whether the IN delivery of F/CT-CRM_{E29H} facilitates protection against live RSV challenge.

Statistical analysis by ANOVA of the results depicted in Figure 9 is as follows:

$p < 0.05$: F/PBS versus F/CT-CRM_{E29H}, F/AlOH, RSV.

$p < 0.05$: Naïve versus F/CT-CRM_{E29H}, F/AlOH, RSV.

$p > 0.05$: F/CT-CRM_{E29H} versus F/AlOH, RSV.

Similar to the results depicted in Figure 7, mice that received IN vaccines containing F/CT-CRM_{E29H} controlled virus replication in the lungs to an extent which was statistically comparable ($p > 0.05$) to that achieved in mice immunized intramuscularly with F/AlOH or IN with live RSV (Log_{10} 1.87 versus Log_{10} 1.99 and Log_{10} 1.94, respectively). IN immunization with F/PBS (first bar), or unimmunized mice (naïve) displayed lung viral titers of Log_{10} 4.5 and Log_{10} 4.3, respectively. Furthermore, these groups had lung virus titers that were found to be statistically elevated ($p < 0.05$) when compared to the virus titers obtained from mice immunized with F/CT-CRM_{E29H}, F/AlOH or live RSV. Therefore, the data support the conclusion that IN instillation of F/CT-CRM_{E29H} protects against infectious RSV challenge.

In a ninth experiment, the anti-F serum antibody response was assessed. The results showed that anti-F protein IgG was significantly increased in mice immunized with F/CT-CRM_{E29H} (0.1 or 1.0 μg) compared to those given F protein delivered in PBS alone (Table 29). In addition, F protein adjuvanted with either 0.1 or 1.0 μg CT-CRM_{E29H} was at least as effective as either F/AlOH (intramuscular) or experimental infection with RSV in stimulating anti-F

protein IgG responses. The magnitude of the anti-F protein antibody titers was dependent on the dose of CT-CRM_{E29H} in the formulation, such that titers were significantly greater in mice that received 1.0 μg CT-CRM_{E29H} versus 0.01 μg. In comparison to F/PBS, both anti-F protein IgG1 and IgG2a titers were augmented with either 0.1 or 1.0 μg CT-CRM_{E29H}. CT-CRM_{E29H} stimulated both type 1 and type 2 immune compartments. Significantly higher serum anti-F protein IgA responses were stimulated by IN immunization with F/CT-CRM_{E29H} (0.1 or 1.0 μg) compared to experimental infection with RSV. In contrast, serum IgA anti-F protein antibodies were not observed in response to F/PBS (IN) or the parenteral administration of F/AlOH (Table 29).

Anti-CT titers also followed a dose-dependent pattern consistent with the anti-F protein titers (Table 29). Statistically equivalent anti-CT titers were observed in sera obtained from mice immunized with either CT-CRM_{E29H} (1.0 μg) or F/CT-CRM_{E29H} (1.0 μg). However, these titers were significantly elevated compared to F/CT-CRM_{E29H} (0.1 or 0.01 μg). In addition, the anti-CT titers in sera of mice immunized with F/CT-CRM_{E29H} (0.1 μg) were statistically heightened compared to titers from mice immunized with F/CT-CRM_{E29H} (0.01 μg). Therefore, the adjuvant effect of CT-CRM_{E29H} for anti-F protein antibody responses is correlated (r = 0.97) with the antibody response to the mutant cholera holotoxin.

In this ninth experiment, mucosal immunity was also assessed. Mucosal IgA was observed only in pooled nasal washes (NW) from mice immunized with either F/CT-CRM_{E29H} (1.0 μg) or F/CT-CRM_{E29H} (0.1 μg) (Table 30). In addition, mice that received IN

immunizations containing purified F protein and CT-CRM_{E29H} (0.01 to 1.0 μg) also had anti-F protein IgA in vaginal washes (VW). F protein-specific IgG was observed in the bronchoalveolar lavage (BAL), VW and/or NW of mice that received F/CT-CRM_{E29H} (0.1 and 1.0 μg), or F/AlOH. In contrast, anti-F protein IgA was not detected in mice immunized IM with F/AlOH.

In a tenth experiment, functional immunity in mice immunized with F protein formulated with CT-CRM_{E29H} was assessed. In the presence of complement, statistically heightened anti-RSV neutralizing antibodies were detected in the sera of mice that had received F protein and either 0.1 or 1.0 μg of CT-CRM_{E29H}, F/AlOH or RSV A2, compared to the administration of F/PBS or CT-CRM_{E29H} alone (Table 31). In the absence of complement, no detectable neutralizing titers were observed in any of the groups ($\log_{10} < 1.3$). Consistent with the serum and mucosal antibody data (Tables 29 and 30), immunization with F/CT-CRM_{E29H} (0.01 μg) was not sufficient to generate anti-RSV neutralizing antibodies.

In an eleventh experiment, immunized mice were challenged two weeks after tertiary immunization, in order to determine the ability of F/CT-CRM_{E29H} to protect against subsequent infection. The results demonstrate that mice immunized with F/CT-CRM_{E29H} (0.1 or 1.0 μg) were protected (Table 32). In comparison to naive mice, or those immunized with F/PBS or CT-CRM_{E29H} alone, the lungs of mice immunized with F protein and either 0.1 or 1.0 μg CT-CRM_{E29H} had significantly reduced virus levels. In addition, significantly reduced virus levels were observed in the nasal tissues of mice immunized with F/CT-CRM_{E29H} (0.1

and 1.0 μg) compared to non-immunized naïve mice or those immunized with F/PBS. In contrast, mice immunized parenterally with F/AlOH displayed reduced viral titers in lung tissue compared to F/PBS immunized mice, but no significant reduction in nasal tissue. Overall, the IN administration of F/ CT-CRM_{E29H} (0.1 or 1.0 μg) was sufficient to generate both local and systemic humoral immune responses that may have contributed to the protection of respiratory tissue against subsequent live RSV challenge.

The data presented in Example 10 have illustrated a viable approach to the development of an IN vaccine for RSV F protein. The data indicate that the production of both humoral and mucosal IgG and IgA is stimulated by the IN delivery of F/CT-CRM_{E29H}. That the antibody titers observed were significant is demonstrated in two ways: First, each of the humoral and mucosal antibody titers that were analyzed in mice immunized with F/CT-CRM_{E29H} were qualitatively similar and quantitatively elevated compared to mice immunized with F/PBS. Second, the elevated titers are translated into a biologically relevant immune response, as indicated by the observed level of protection displayed in Figures 7 and 9. Immunization with F/CT-CRM_{E29H} significantly enhanced protection against live RSV challenge compared to immunization with F/PBS or PBS/CT-CRM_{E29H}.

Collectively, the data suggest a mechanism involving the neutralization of infectious virus by either mucosal or humoral immunoglobulins, that are stimulated in response to the IN immunization protocol containing F/CT-CRM_{E29H}.

Mice were immunized with another viral antigen, rotavirus, in the form of recombinantly-

expressed VP2 and VP6 proteins. Recombinant baculovirus vectors expressing SA11 rotavirus VP2 and VP6 were constructed as described previously (28); it is known that recombinantly-expressed rotavirus structural proteins self-assemble into particles that are morphogenically indistinguishable from virions. The proteins so expressed co-assembled into 2/6-virus-like particles (VLPs).

Inbred BALB/c mice with a homogeneous genetic background were used, anticipating that they all would be responsive to immunization and thus clarify the profile of systemic and mucosal immunoglobulins to VLPs alone and when combined with CT-CRM_{E29H} adjuvant. Genetically heterogeneous outbred CD-1 mice were also used to determine the effect of genetic diversity on the factors involved in induction of immunity and protection.

Sera of all immunized BALB/c and CD-1 mice, except for two unresponsive orally immunized CD-1 mice, contained antibodies to both VP2 and VP6. Pre-immunization sera as well as sera of unimmunized mice detected no viral antigen in mock and or VP2-6 baculovirus infected cells. 2/6-VLP immunized sera or mAbs specific for VP6 and VP2 exposed to uninfected cells also demonstrated no reactivity (data not shown). Immunogenicity of 2/6-VLP was also confirmed by Western blot analysis using pre-challenged and immunized serum from each mouse against 2/6-VLP as well as SA11 strain of rotavirus (data not shown).

Patterns of rotavirus-specific serum IgG, IgM and IgA in the group immunized IN with 2/6-VLPs alone were similar to those in the group immunized with 2/6-VLPs and CT-CRM_{E29H} (Figure 10). However, week 13 levels of the three serum antibody isotypes were significantly higher in the animals receiving 2/6-VLPs

together with CT-CRM_{E29H} (Figure 10) (P=0, P=0.004, and P=0.02 for IgG, IgM and IgA respectively). This indicated that CT-CRM_{E29H} had significantly enhanced humoral responses to 2/6-VLPs. Week 13 was selected as indicative of antibody levels generated following two immunizations but prior to challenge.

As shown in Figure 11A, BALB/c mice given VLPs IN produced a greater rotavirus specific systemic IgG response than those immunized orally. Statistically significant differences in serum IgG titers between the two groups were evident at 13 weeks (P=0). Similarly, serum IgM levels were higher in the IN group as opposed to the oral group when pre-challenge (week 13) values were analyzed (P=0) (Figure 11B). Serum IgM levels peaked by week 2 and decreased by week 4 in the IN and mixed groups, whereas in the oral group, low but relatively constant levels of serum IgM were detected throughout the study. Peak serum IgA in orally and IN immunized animals occurred on week 4. No significant differences in serum IgA levels distinguished the three experimental groups when examined at week 13 (Figure 11C). Overall, IN immunization generated higher levels of systemic IgG and IgM responses than oral immunization. Induction of significantly high levels of IgG and IgM in the IN group support the concept that IN immunization may be the preferred route for administration of future vaccine candidates (33). Thus, IN immunization leading to strong systemic neutralizing responses could be effective against viral pathogens that penetrate mucosal barriers.

Both IgG1 and IgG2a were found in the serum of the IN and the orally immunized BALB/c mice (Figure 12). The IN group had statistically significantly higher levels of both IgG subclasses compared to the

oral group (IgG1, P=0.005; IgG2a, P=0.05). However, there were no significant differences between subclasses within each group. These data confirm the utility of IN immunization for effective induction of both T-helper pathways (TH-1 and TH-2).

The peak level of fecal IgA for all three experimental groups occurred four weeks after the first immunization (Figure 13A), coinciding temporally with the time at which serum IgA levels were maximal in orally and IN immunized animals (Figure 11C). No statistically significant differences were found among the three immunization protocols when the pre-challenge fecal IgA levels were examined (Figure 13A). Fecal IgG also was maximal on week 4 (Figure 13B); however, the IN group had significantly more IgG on week 13 compared to oral or mixed groups (P=0 and P=0.002, respectively). In general, all 2/6-VLP immunized mice produced serum IgG, IgM and IgA, as well as fecal IgG and IgA. No fecal IgM was detected in any of the animals.

All CD-1 mice receiving 2/6-VLPs IN (n=4) and two of four mice immunized orally produced rotavirus-specific antibody responses (data not shown). To determine the profile of antibody responses more precisely, serum and fecal samples were analyzed weekly for 26 weeks. The induction pattern of serum and fecal antibodies in CD-1 mice was similar to that in the BALB/c mice (Figures 11 and 13).

In BALB/c mice, two IN immunizations with 2/6-VLPs and CT-CRM_{E29H} proved protective (PRAS=98.7%), in contrast to IN immunization with 2/6-VLPs alone (PRAS=39%) (P=0.007) (Figure 14A). The mixed immunization, IN followed by oral immunization, protected mice to an extent similar to oral and IN groups, indicating that in BALB/c mice mixed

immunization was also effective. This demonstrated the significant augmentation in protective immune responses due to CT-CRM_{E29H}. BALB/c mice in all three immunization groups showed nearly complete protection from the challenge. PRAS was 99.6%, 98.8% and 98.8% for the oral, IN and the mixed groups, respectively (Figure 14B). The unimmunized control group shed significantly more viral antigen than the three immunized groups (P=0). There were no significant differences between PRAS values for the three immunized groups.

IN immunization induced both systemic and mucosal responses in all immunized CD-1 mice and protected these animals (PRAS=97.9%) (Figure 14C). Only two of four orally immunized CD-1 mice showed systemic and mucosal antibody responses and protection (2 of 3, PRAS 65.8%) (Figure 14C); in contrast, all orally immunized BALB/c mice showed mucosal and systemic responses and were protected. Notably, the CD-1 mouse that did not produce an immune response was not protected from infection, whereas immune-responsive mice were protected (Figure 14C). One mouse in the oral group, with no antibody response to immunization, died prior to the challenge. Two CD-1 mice were used as controls to reduce the number of samples in the analysis of antibody responses. However, statistical analysis clearly showed that the protection results were significant (P=0, at 95% confidence level). The oral immunization experiment was repeated with CD-1 mice under the same conditions, except that animals were challenged on week 13 rather than 26. Using four mice in the immunized groups and five mice as controls, a similar protection level was observed (PRAS=71.2%) (data not shown). Taken together, these results support those recently published by O Neal et al.

(34,35), suggesting that in CD-1 mice intranasal immunization is more effective than oral immunization.

In view of this demonstrated utility of CT-CRM_{E29H} as a vaccine adjuvant, production of suitable quantities of this material is desirable. Using pIIB29H (described in Example 1), several attempts were made to express CT-CRM_{E29H} in *E.coli*. The resulting yield of purified CT-CRM_{E29H} holotoxin was approximately 50μg per liter of culture medium. Initial attempts to increase CT-CRM_{E29H} yield via modifications to the original plasmid, pIIB29H, to create plasmid pPX2492 (see Example 1), showed little or no effect. A moderate increase in yield was achieved through co-expression of pIIB29H, and derivatives, with *Vibrio cholerae* DsbA and *E. coli* RpoH. Co-expression and purification modifications increased the yield of CT-CRM_{E29H} to approximately 2 mg per liter.

In order to increase the expression of CT-CRM_{E29H}, the lactose inducible promoter was replaced with an arabinose inducible promoter (Invitrogen Corporation, Carlsbad, CA), which was operatively linked to the DNA sequence encoding CT-CRM_{E29H}. During cloning it was determined that plasmid pIIB29H contained a *ctxA* gene from *Vibrio cholerae* strain 569B, linked to a *ctxB* gene from *V.c.* strain 2125. Cross alignment of these genes indicated seven base substitutions between the two *ctxB* genes and a single base change between the *ctxA* genes. Several of these base substitutions led to amino acid changes in the mature subunits. Of special note is the substitution between the *ctxA* genes which leads to an amino acid change within the A-2 portion, or the holotoxin assembly domain of the A subunit. It was not known whether the heterogeneity between these genes had a

negative impact on toxin expression or holotoxin assembly; however, it was thought preferable from an evolutionary standpoint that both toxin subunit genes originate from the same source. As such, both the *ctxA* and *ctxB* genes used in the construction of the arabinose inducible system originated from *Vibrio cholerae* strain 569B. The construction of plasmid pPX7490 is described in Example 12. Production of CT-CRM_{E29H} from pPX7490 is approximately 30 mg of purified material per liter of culture.

The invention further relates to plasmids containing isolated and purified DNA sequences comprising DNA sequences which encode an immunogenic mutant cholera holotoxin having a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the cholera holotoxin, and wherein such a DNA sequence is operatively linked to an arabinose inducible promoter, as well as to suitable host cells transformed, transduced or transfected with such plasmids by conventional techniques.

A variety of host cell-plasmid vector systems are used to express the immunogenic mutant cholera holotoxin. The vector system, which preferably includes the arabinose inducible promoter, is compatible with the host cell used. Suitable host cells include bacteria transformed with plasmid DNA, cosmid DNA or bacteriophage DNA; viruses such as vaccinia virus and adenovirus; yeast such as Pichia cells; insect cells such as Sf9 or Sf21 cells; or mammalian cell lines such as Chinese hamster ovary cells; as well as other conventional organisms.

A variety of conventional transcriptional and translational elements can be used for the host cell-vector system. The DNA encoding the CT-CRM is inserted into an expression system, and the promoter (preferably

the arabinose inducible promoter) and other control elements are ligated into specific sites within the vector, so that when the plasmid vector is inserted into a host cell (by transformation, transduction or transfection, depending on the host cell-vector system used), the DNA encoding the CT-CRM is expressed by the host cell.

The immunogenic mutant cholera holotoxin is produced by transforming, transducing or transfecting a host cell with a plasmid described above and culturing the host cell under conditions which permit the expression of said recombinant immunogenic detoxified protein by the host cell.

Although this invention is exemplified by a CT-CRM mutant having a histidine at amino acid 29, other nonconservative mutations of the wild-type glutamic acid residue are also within the scope of this invention. Glutamic acid is an acidic (negatively charged) molecule. Therefore, a nonconservative mutation will be one in which a substitution is made to an amino acid other than aspartic acid, which is also an acidic molecule. Suitable alternative amino acids include the amino acids lysine and arginine which, like histidine, are basic (positively charged) molecules. Suitable alternative amino acids further include the amino acids with nonpolar functional groups such as alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan and valine, and the amino acids with uncharged polar functional groups such as asparagine, cysteine, glutamine, glycine, serine, threonine and tyrosine.

An effective amount of the mutant cholera holotoxin, wherein the holotoxin has reduced toxicity compared to a wild-type cholera holotoxin and has a substitution other than aspartic acid for the glutamic

acid at position 29 of the A subunit of the cholera holotoxin, in combination with a selected antigen from a pathogenic bacterium, virus, fungus or parasite, is used to prepare an antigenic composition, wherein said holotoxin enhances the immune response in a vertebrate host to said antigen.

The antigenic compositions of this invention also comprise CT-CRM containing at least one additional mutation at a position other than at amino acid residue 29. International application WO 93/13202 (36), which is hereby incorporated by reference, describes a series of mutations in the A subunit which serve to reduce the toxicity of the cholera holotoxin. These mutations include making substitutions for the arginine at amino acid 7, the aspartic acid at position 9, the arginine at position 11, the histidine at position 44, the valine at position 53, the arginine at position 54, the serine at position 61, the serine at position 63, the histidine at position 70, the valine at position 97, the tyrosine at position 104, the proline at position 106, the histidine at position 107, the glutamic acid at position 110, the glutamic acid at position 112, the serine at position 114, the tryptophan at position 127, the arginine at position 146 and the arginine at position 192. The nucleotide sequence encoding the A subunit of the cholera holotoxin is set forth in International application WO 93/13202. International application WO 98/42375 (37) which is hereby incorporated by reference, describes making a substitution for the serine at amino acid 109 in the A subunit, which serves to reduce the toxicity of the cholera holotoxin. Therefore, using conventional techniques, mutations at one or more of these additional positions are generated.

The antigenic compositions of this invention are administered to a human or non-human vertebrate by a variety of routes, including, but not limited to, intranasal, oral, vaginal, rectal, parenteral, intradermal, transdermal (see, e.g., International application WO 98/20734 (38), which is hereby incorporated by reference), intramuscular, intraperitoneal, subcutaneous, intravenous and intraarterial. The amount of the antigen component or components of the antigenic composition will vary depending upon the identity of the antigen, as well as upon the age, weight and medical condition of the host, as well as on the method of administration. Again, suitable doses are readily determined by persons skilled in the art. It is preferable, although not required, that the antigen and the mutant CT be administered at the same time. The number of doses and the dosage regimen for the antigenic composition are also readily determined by persons skilled in the art. Protection may be conferred by a single dose of the antigenic composition, or may require the administration of several doses, in addition to booster doses at later times to maintain protection. In some instances, the adjuvant property of the mutant CT may reduce the number of doses needed or the time course of the dosage regimen.

The antigenic compositions of this invention may comprise further adjuvants in addition to CT-CRM_{E29H}. Examples of such adjuvants include, but are not limited to, Stimulon™ QS-21 (Aquila Biopharmaceuticals, Inc., Framingham, MA), MPL™ (3-O-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, MT), aluminum phosphate, aluminum hydroxide and IL-12 (Genetics Institute, Cambridge, MA). The antigenic compositions may also be

mixed with immunologically acceptable diluents or carriers in a conventional manner.

The immunogenic mutant cholera holotoxin of this invention is suitable for use as an adjuvant in antigenic compositions containing a wide variety of antigens from a wide variety of pathogenic microorganisms, including but not limited to those from bacteria, viruses, fungi or parasitic microorganisms which infect humans and non-human vertebrates. The antigen may comprise a whole cell or virus, or one or more saccharides, proteins, protein subunits or fragments, poly- or oligonucleotides, or other macromolecular components. If desired, the antigenic compositions may contain more than one antigen from the same or different pathogenic microorganisms.

Desirable bacterial vaccines including the CT-CRM mutants as an adjuvant include those directed to the prevention and/or treatment of disease caused by, without limitation, *Haemophilus influenzae* (both typable and nontypable), *Haemophilus somnus*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus faecalis*, *Helicobacter pylori*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Chlamydia* psittaci, *Bordetella pertussis*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella choleraesuis*, *Escherichia coli*, *Shigella*, *Vibrio cholerae*, *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, *Mycobacterium avium- Mycobacterium intracellulare* complex, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus aureus*, *Clostridium tetani*, *Leptospira interrogans*, *Borrelia burgdorferi*, *Pasteurella haemolytica*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* and *Mycoplasma gallisepticum*.

Desirable viral vaccines including the CT-CRM mutants as an adjuvant include those directed to the prevention and/or treatment of disease caused by, without limitation, Respiratory syncytial virus, Parainfluenza virus types 1-3, Influenza virus, Herpes simplex virus, Human cytomegalovirus, Human immunodeficiency virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Human papillomavirus, poliovirus, rotavirus, caliciviruses, Measles virus, Mumps virus, Rubella virus, adenovirus, rabies virus, canine distemper virus, coronavirus, parvovirus, infectious rhinotracheitis viruses, feline leukemia virus, feline infectious peritonitis virus, avian infectious bursal disease virus, Newcastle disease virus, Marek's disease virus, porcine respiratory and reproductive syndrome virus, equine arteritis virus and various Encephalitis viruses.

Desirable vaccines against fungal pathogens including the CT-CRM mutants as an adjuvant include those directed to the prevention and/or treatment of disease caused by, without limitation, *Aspergillis*, *Blastomyces*, *Candida*, *Coccidiodes*, *Cryptococcus* and *Histoplasma*.

Desirable vaccines against parasites including the CT-CRM mutants as an adjuvant include those directed to the prevention and/or treatment of disease caused by, without limitation, *Leishmania major*, *Ascaris*, *Trichuris*, *Giardia*, *Schistosoma*, *Cryptosporidium*, *Trichomonas*, *Toxoplasma gondii* and *Pneumocystis carinii*.

The CT-CRM mutants are also suitable for inclusion as an adjuvant in polynucleotide vaccines (also known as DNA vaccines). Such vaccines may further include facilitating agents such as bupivicaine

(see U.S. Patent Number 5,593,972 (39), which is hereby incorporated by reference).

CT-CRM_{E29H} was compared to wild-type CT as an adjuvant for the administration of plasmid DNA (pDNA) encoding the full length glycoprotein D of herpes simplex virus (HSV) type 2 (gD2), formulated with bupivicaine (40). The results indicated that BALB/c mice which received CT-CRM_{E29H} along with pDNA vaccine for HSV-2 by the intradermal route generated a higher average cellular response than those that received pDNA HSV gD2 vaccine by itself by the intradermal route (Table 34). In addition, the average antibody response in serum for mice which received the pDNA HSV gD2 vaccine along with CT-CRM_{E29H} was approximately at the same level as that seen for mice which received the pDNA HSV gD2 vaccine without adjuvant (Table 35).

Similarly, the pDNA HSV gD2 vaccine generated a gD2-specific antibody response in vaginal wash samples at levels that were comparable to those seen following the delivery of non-adjuvanted vaccine by intradermal or intramuscular routes (Table 36).

Mice immunized with the pDNA HSV gD2 vaccine adjuvanted with CT-CRM_{E29H} or CT and delivered by the intradermal route generated substantially higher levels of gamma interferon than mice which received the pDNA HSV-gD2 vaccine without adjuvant (Table 37). Mice which received the CT-CRM_{E29H} also generated IL-5.

Thus, CT-CRM_{E29H} enhanced proliferative and gamma interferon responses when administered with a plasmid DNA vaccine against HSV.

In order that this invention may be better understood, the following examples are set forth. The examples are for the purpose of illustration only and

are not to be construed as limiting the scope of the invention.

Examples

Example 1
Expression of CT Mutants

Bacterial strains, plasmids and growth conditions

E. coli TG1 (Amersham-Pharmacia Biotech, Piscataway, NJ), and TX1, a nalidixic acid-resistant derivative of TG1, carrying FTc,lacI^q from XL1 blue (Stratagene, LaJolla, CA; (41)) and CJ236(FTc, lacI^q) (Bio-Rad, Hercules, CA) were used as hosts for cloning recombinant plasmids and expression of mutated proteins. Plasmid-containing strains were maintained on LB agar plates with antibiotics as required (ampicillin, 50 μg/ml; kanamycin 25 μg/ml; tetracycline 10 μg/ml). A complete CT operon from *V. cholerae* 0395 was subcloned into the phagemid vector pSKII$^-$, under the control of the *lac* promoter, to create the IPTG inducible plasmid designated pMGJ67 (42).

Mutagenesis of *ctx*A gene

The method of Kunkel (43) was used to select for oligonucleotide-derived mutants created in plasmid pMGJ67. The oligonucleotides used to generate the five mutant CT-CRMs are described in Table 1.

Table 1
Sequence of Oligonucleotides Introduced into *ctx*A

Substitution	Oligonucleotide Sequence[a]	
R7K	AAGTTATATAAGGCAGATTC	(SEQ ID NO:1)
R11K	CAGATTCTAAACCTCCTG	(SEQ ID NO:2)
E29H	GACAGAGTNAGTACTTTGACCG	(SEQ ID NO:3)
E110D	CAGATGAKCAAGAKGTTTCTGC	(SEQ ID NO:4)
E112D	CAGATGAKCAAGAKGTTTCTGC	(SEQ ID NO:5)

[a] Altered bases are underlined; N=any base; K=T or G.

Briefly, each single-stranded oligonucleotide was phosphorylated and used to direct second strand synthesis on a uracil-containing single-stranded DNA template rescued from the *E. coli* dut ung strain CJ236(F'Tc, pMGJ67). Following ligation and transformation of ung$^+$ strain TX1, single-stranded DNA was rescued from Amp^R transformants and sequenced by the dideoxy chain termination method (44).

Construction of the Plasmid Encoding CT-CRM$_{E29H}$

The plasmid encoding CT-CRM$_{E29H}$ is designated pIIB29H. The plasmid contains the polycistron of *V. cholerae* genes *ctx*A and *ctx*B which encode CT. The *ctx*A gene in this plasmid was mutagenized as described above to encode a histidine at amino acid position 29 of CT-A. The wild-type polycistron was also altered by removing the native ToxR inducible promoter and replacing it with a lactose inducible promoter. Furthermore, the regions encoding the *ctx*A and *ctx*B

signal sequences were replaced with the signal sequence-encoding region of *E. coli* LT (LTIIb-B leader) in order to promote secretion of CT-CRM_{E29H}. The plasmid pIIB29H was then modified in an attempt to increase the expression of CT-CRM_{E29H}. The resulting plasmid, designated pPX2492, contained synthetic Shine Dalgarno sequences upstream of each of *ctx*A and *ctx*B. The two genes are genetically separated in pPX2492, unlike in *V. cholerae*, where the genes overlap. The two genes also have the LTIIb-B leader sequence upstream of each.

Expression of mutant ctxA alleles

Production of each variant holotoxin was tested in 5 ml cultures of TB medium (45) in 125 ml Erlenmeyer flasks at 37°C with shaking (200 rpm). Logarithmic phase cells (A_{600} = 0.8-1.0) were induced by the addition of IPTG to 0.4 mM, followed by growth overnight. Polymyxin B was added to 1 mg/ml, followed by incubation for 10 minutes at 37°C. Cells were removed by centrifugation, and the supernatants were assayed to determine the concentrations of holotoxin and B pentamer as described below.

Specifically, the production of CT-CRM_{E29H} in *E. coli* involves the co-expression of the genes *rpo*H from *E. coli* and *dsb*A from *V. cholerae*. These gene products participate in the conformational maturation of both the A and B subunits of CT.

Example 2
The GM1 Binding Assay for Intact Holotoxin

The CT-CRMs were examined in a ganglioside GM_1-dependent solid phase radioimmunoassay (42) to determine whether intact holotoxin was present after purification. An enzyme-linked immunosorbent assay (ELISA) was used where ELISA plate microwells were coated overnight at 4°C with ganglioside GM_1 (10 µg/ml). Thereafter, the following reagents were added in sequence with an interval of one hour incubation at room temperature: CT-CRMs (titrated from 3 µg/ml to 0.00137 µg/ml), 100 µl of rabbit anti-CT-A sera (1:1,000), and alkaline phosphatase conjugated goat anti-rabbit antibody (1:2,000). To visualize the reaction, 100 µl of *p*-nitrophenyl phosphate at 1 µg/ml in diethanolamine was added and incubated for 30 minutes. The reaction was stopped by adding 100 µl of 2 N NaOH and immediately read by a Microelisa autoreader. When compared to wild-type CT, the data indicated that the CT-CRMs with amino acid substitutions at positions 7, 29, 110, or 112 were intact holotoxins (Figure 1). The results implied, however, that a portion of purified CT-CRM_{R11K} did not appear to be a holotoxin.

Example 3
Y-1 Adrenal Cell Assay for Residual Toxicity of CT-CRMs

The mutant CT-CRMs were compared several times with wild-type holotoxin for toxicity in the mouse Y-1 adrenal tumor cell assay. Y-1 adrenal cells (ATCC CCL-79) were seeded in 96-well flat-bottom plates at a concentration of 10^4 cells per well. Thereafter,

three-fold serial dilutions of CT-CRMs were added to the tumor cells and incubated at 37°C (5% CO_2) for 18 hours. The cells were then examined by light microscopy for evidence of toxicity (cell rounding). The endpoint titer was defined as the minimum concentration of toxin required to give greater than 50% cell rounding. The percent of residual toxicity is calculated using the endpoint titer of wild-type CT divided by the titer elicited by CT-CRM multiplied by 100. Table 2 depicts the residual toxicity of several purified mutant holotoxins tested in the Y-1 adrenal cell assay.

Table 2

The toxicity for Y-1 adrenal cells

Y-1 Adrenal Cell Assay	
CT-CRM	% Residual Toxicity
E112D	0.13
E112D	0.13
R11K	0.04
R7K	0.04
E110D	0.13
E110D	0.40
E29H	1.20
CT-B	1.20
CT	100.00

Example 4
Patent Mouse Gut Weight Assay

In this assay, 10 μg of wild-type CT or CT-CRM_{E29H} was administered intragastrically to each group of BALB/c mice (three mice per group). Intestines were removed carefully three hours later and weighed. The results are presented in Table 3. Data are presented as the mean gut/carcass weight ratio per group.

Table 3
Toxicity of CT-CRM_{E29H}

Assay	CT	CT- CRM_{E29H}	PBS
Mouse Gut Weight (gut/carcass ratio)	0.13 ± 0.01	0.09 ± 0.01[a]	0.08 ± 0.007

[a] $p < 0.05$ compared to wild-type CT control, $p > 0.05$ compared to PBS.

Example 5
The ADP-ribosyltransferase Assay

NAD^+:agmatine ADP-ribosyltransferase activity was measured as the release of [carbonyl-^{14}C] nicotinamide from radiolabeled NAD^+. Briefly, CT and CT-CRMs were trypsin activated and incubated for 30 minutes at 30°C with 50 mM glycine/20 mM dithiothreitol in TEAN buffer (Tris™/EDTA/sodium azide/sodium chloride) (pH 8.0). Thereafter, the following materials were added to the reaction: 0.1 mg of soybean trypsin inhibitor, 50 mM potassium phosphate, 10 mM agmatine, 20 mM dithiothreitol, 10 mM magnesium

chloride, 100 μM GTP, 3 mM dimyristoylphosphatidylcholine, 0.2% cholate, 0.03 mg of ovalbumin, 100 μM [adenine-U-^{14}C]NAD (DuPont NEN™, Boston, MA) and water to a final volume of 300 μl. After incubation for 90 minutes at 30° C, 100 μl samples were applied to columns (0.64 x 5 cm) of AG1-X2 (Bio-Rad) which were washed five times with 1.0 ml of distilled/deionized H_2O. Eluates containing [^{14}C]ADP-ribosylagmatine were collected for radioassay. Mean recovery of ^{14}C in the eluate is expressed as percentage of that applied to column. The results are presented in Table 4.

Table 4

NAD:Agmatine ADP-Ribosyltransferase Activity

Adjuvant	ADP-ribosylagmatine formed (nmol/hr/μg protein)	% ADP-ribosylation activity
CT, 10 μg	57.1	100
E29H, 10 μg	6.7	11.7
E110D, 10 μg	0.4	0.7
E112D, 10 μg	0.9	1.6
R7K, 10 μg	0.4	0.7
R11K, 10 μg	0.4	0.7

Example 6

The Immune Responses of BALB/c Mice Immunized with Recombinant (r) P4 and P6 Outer Membrane Proteins of Nontypable *Haemophilus influenzae* (NTHi)

In a first experiment, five BALB/c mice per group were immunized intranasally on days 0, 21 and 35 with a 10 µl dose containing 5 µg rP4 or 10 µg rP6, plus 1 µg of the adjuvant as indicated in Tables 5 and 6 (one group did not receive adjuvant). The anti-rP4 IgG antibody titers were determined by ELISA on pooled samples collected at days 0, 21, 35 and 48 and the results shown in Table 5. The anti-rP6 IgG antibody titers were separately determined by ELISA on pooled samples collected at days 0, 21, 35 and 48 and the results shown in Table 6. The mucosal antibody responses to rP4 were also measured two weeks after the last immunization (day 49). Table 7 sets forth the IgA and IgG titers from nasal, bronchoalveolar and vaginal washes, respectively.

In a second experiment, five BALB/c mice per group were immunized intranasally on days 0, 21 and 35 with a 30 µl dose containing 5 µg rP4 or 10 µg rP6, plus ascending doses of CT-CRM_{E29H} as indicated in Table 8 (other groups each received CT or CT-B; one group received no adjuvant). The serum anti-rP4 IgA and IgG antibody titers were determined by ELISA on pooled samples collected at days 21, 35 and 48 and the results shown in Table 8. The IgA and IgG titers from bronchoalveolar and vaginal washes on day 49 were also determined and are shown in Table 8.

Table 5

The systemic humoral immune responses of BALB/c mice immunized[a] with recombinant P4 and P6 proteins[b] formulated with mutant cholera holotoxins

	Serum Anti-Recombinant P4 IgG Antibody Titers[c]			
Adjuvant[d]	Day 0	Day 21	Day 35	Day 48
None	1,157	1,277	1,893	1,968
CT	751	1,657	17,589	45,885
CT-B	1,111	1,118	6,917	70,578
E29H	1,052	1,539	11,917	95,922
E110D	1,243	1,313	6,886	83,058
E112D	1,400	1,520	9,280	41,485
R7K	2,546	1,771	3,311	40,936
R11K	1,289	1,391	3,428	23,631

[a] The mice were immunized intranasally (IN, 10 μl volume) on days 0, 21 and 35.

[b] Recombinant P4 and P6 proteins were administered at 5 and 10 μg per dose respectively.

[c] Anti-recombinant P4 IgG antibody titers were determined by ELISA on pooled samples collected at the denoted times. There were 5 mice per group.

[d] CT and CT mutants were administered at 1 μg per dose.

Table 6

The systemic humoral immune responses of BALB/c mice immunized[a] with recombinant P4 and P6 proteins[b] formulated with mutant cholera holotoxins

	Serum Anti-Native P6 IgG Antibody Titers[c]			
Adjuvant[d]	Day 0	Day 21	Day 35	Day 48
None	< 100	< 100	< 100	< 100
CT	< 100	< 100	9,644	54,821
CT-B	< 100	< 100	875	7,399
E29H	< 100	< 100	3,472	19,638
E110D	< 100	< 100	3,666	22,415
E112D	< 100	< 100	426	9,538
R7K	< 100	< 100	529	3,904
R11K	< 100	< 100	248	3,763

[a] The mice were immunized intranasally (IN, 10 μl volume) on days 0, 21 and 35.

[b] Recombinant P4 and P6 proteins were administered at 5 and 10 μg per dose respectively.

[c] Anti-recombinant P6 IgG antibody titers were determined by ELISA on pooled samples collected at the denoted times. There were 5 mice per group.

[d] CT and CT mutants were administered at 1 μg per dose.

Table 7

The mucosal antibody responses of BALB/c mice immunized[a] with recombinant P4 and P6 proteins[b] formulated with mutant cholera holotoxins

	Anti-Recombinant P4 Antibody Titers[c]					
	NW[d]		BAW[d]		VW[d]	
Adjuvant[e]	IgA	IgG	IgA	IgG	IgA	IgG
None	< 5	< 5	< 5	< 5	< 50	< 50
CT	< 5	< 5	< 5	56	54	< 50
CT-B	< 5	< 5	< 5	99	< 50	< 50
E29H	< 5	< 5	< 5	176	63	< 50
E110D	< 5	< 5	< 5	144	< 50	98
E112D	11	< 5	< 5	48	564	58
R7K	< 5	< 5	< 5	56	< 50	< 50
R11K	6	< 5	< 5	34	223	< 50

[a] The mice were immunized intranasally (IN, 10 μl volume) on days 0, 21 and 35.

[b] Recombinant P4 and P6 proteins were administered at 5 and 10 μg per dose respectively.

[c] Anti-recombinant P4 IgG and IgA antibody titers were determined by ELISA on pooled samples collected 2 weeks

after the last immunization (day 49). There were 5 mice per group.

[d] NW, BAW, and VW denote nasal wash, bronchoalveolar wash, and vaginal wash respectively.

[e] CT and CT mutants were administered at 1 μg per dose.

Table 8

The effect of ascending doses of CT-CRM_{E29H} on the generation of local and systemic immune responses against the recombinant P4 and P6 proteins of *Haemophilus influenzae*

Anti-Recombinant P4 Antibody Titers[a]

	Sera						Mucosal Wash Fluids[b]			
	Day 21		Day 35		Day 48		BAW		VW	
Adjuvant	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
none	189	219	204	3,833	334	17,269	27	26	493	146
1 μg CT	145	741	1,803	67,470	5,855	495,735	864	2,279	1,446	1,934
10 μg CT-B	206	2,531	650	34,047	5,838	989,806	622	3,674	1,397	884
1 μg CT-CRM_{E29H}	171	2,640	1,021	59,031	8,643	588,586	845	3,180	1,898	1,479
10 μg CT-CRM_{E29H}	<100	498	<100	6,427	397	84,176	25	175	122	31
30 μg CT-CRM_{E29H}	119	622	801	14,151	2,194	161,187	67	568	215	116

[a] The mice were immunized intranasally (IN, 30 μl volume) on days 0, 21 and 35 with recombinant P4 (5 μg) and P6 (10 μg) proteins. Anti-recombinant P4 IgG and

Table 8 (continued)

IgA antibody titers were determined by ELISA on pooled samples collected at the indicated times. There were 5 mice per group.

[b] BAW and VW denote bronchoalveolar wash, and vaginal wash respectively.

Example 7

The Immune Responses of BALB/c Mice Immunized with the Native Hap_S Protein of NTHi

NTHi strain P860295 (46) was obtained from Dr. Charles Brinton, University of Pittsburgh. It was obtained from the nasopharynx of a child with NTHi induced otitis media. NTHi strain TN106 (47) was obtained from Dr. Eric Hansen, University of Texas Southwestern Medical Center at Dallas. A streptomycin resistant mutant of TN106 was derived by selection on BHI-XV plates containing 100 μg/ml of streptomycin (Sigma, St. Louis, MO). This mutant was passaged twice in the nasopharynx of Balb/c mice and frozen as strain TN106.P2.

The Hap_S protein from NTHi strain P860295 was purified as follows. NTHi strain P860295 was grown in BHI-XV media for 18 hours at 35°C with aeration. The bacterial cells were pelleted by centrifugation, 10K x g at 4°C, and discarded. The supernatant was brought to 60% saturation with solid $(NH_4)_2SO_4$, held at room temperature for 2-3 hours, and the precipitate was collected by centrifugation. The precipitate was dissolved in 50 mM sodium phosphate buffer, pH 5.8, 1 mM EDTA, 50 mM NaCl (Buffer 1), and was dialyzed at 4°C against the above buffer. A 10 ml bed volume CM Sepharose™ column (Pharmacia, Piscataway, NJ) was equilibrated with Buffer 1, and 30 ml of the above soluble material was loaded onto the column at a flow rate of 1 ml/min. The column was washed with Buffer 1 until the OD_{280} reached baseline. The fall-through material was discarded. Bound proteins were eluted from the resin using a three step gradient: (1) sodium phosphate buffer, pH 7.0, 1mM EDTA, 50 mM NaCl; (2) sodium phosphate buffer, pH 8.0, 1 mM EDTA, 50 mM NaCl;

and (3) sodium phosphate buffer, pH 8.0, 0.5 M NaCl, 1 mM EDTA. Proteins eluted in each step were pooled and saved for analysis. SDS-PAGE (48) analysis of pools indicated that the Hap_S protein eluted in gradient steps 2 and 3. These pools contained highly purified Hap_S and were combined.

Six week old, female Balb/c mice (ten per group) were then immunized IN with Hap_S purified from NTHi strain P860295. The Hap_S protein was diluted in D-PBS to 5 or 15 μg/40 μl with or without CT-CRM_{E29H}. Where used, the CT-CRM_{E29H} was used at a dosage of 0.1 μg/mouse. Control formulations containing CT-CRM_{E29H} in D-PBS, D-PBS alone and formalin fixed TN106.P2 (the NTHi challenge strain) were also administered to the mice in 40 μl volumes.

Prior to IN immunization, mice were anesthetized and then immunized by intranasal inoculation of 20 μl/nostril from a pipette. The pipette was held so the tip touched the opening of the nostril and the formulation was automatically drawn into the nostril during breathing. The mice were placed in a supine position so noses were not touching anything after administration of the formulation or the challenge. The mice were immunized at weeks 0, 1, 3, and 5. Sera were drawn at week 7. The results are shown in Table 9.

Table 9
Systemic humoral immune response in Balb/c mice after intranasal immunization with Hap_s admixed with or without $CT\text{-}CRM_{E29H}$

Immunogen	Dose (μg)	Adjuvant	Anti-Hap_s IgG ELISA
Hap	5	-	1,604
Hap	15	-	5,204
Hap	5	CT-E29H	4,653
Hap	15	CT-E29H	15,111
-	-	CT-E29H	<500
1xPBS	-	-	<500
Formalin Fixed TN106.P2		-	<500

Example 8

The Immune Responses of BALB/c and C57Bl/6 Mice Immunized with the Recombinant (r) Urease Protein of *Helicobacter pylori*

In a first experiment, five BALB/c mice per group were immunized as follows: Seven groups were immunized intragastrically on days 0, 2, 14 and 16 with 100 µg rUrease plus 10 µg of the adjuvant as indicated in Tables 10-12. One group was immunized with 10 µg rUrease subcutaneously in the rump; another group was immunized with 10 µg rUrease subcutaneously in the neck; both groups also received 20 µg of Stimulon™ QS-21 as an adjuvant on days 0 and 16. The anti-rUrease antibody titers were determined by ELISA on pooled

samples collected on day 28. The IgG results are shown in Table 10 and the IgA results are shown in Table 11. The mucosal antibody responses to rUrease were also measured on day 29. Table 12 sets forth the IgA and IgG titers from bronchoalveolar and vaginal washes, respectively.

In a second experiment, the ability of rUrease plus adjuvant to protect mice against a challenge with *H. felis* was assessed. Ten BALB/c mice per group were immunized as follows: Two groups were immunized intragastrically on days 0, 2, 14 and 16 with 100 µg rUrease plus 10 µg of the adjuvant as indicated in Table 13; a control group received PBS instead of rUrease plus 10 µg of adjuvant. One group was immunized subcutaneously on days 0 and 16 with 10 µg rUrease plus 20 µg of Stimulon™ QS-21. The anti-rUrease antibody titers were determined by ELISA on pooled samples collected on day 28. The mice were also challenged with three doses of 10^8 *H. felis* on days 29, 31 and 34 and were assayed for protection on day 44. Protection was assessed by the rapid urease test. In the rapid urease test, one-half stomach was incubated at 37°C for five hours in 0.5 ml of the urease test medium containing 2% of urea and phenol red, a pH indicator, at 7 µg/ml. Urease activity generates ammonium and bicarbonate from urea, thus raising the pH and inducing a colorimetric change of the solution with a higher absorbance at 550 nm. The level of urease activity was measured by spectrophotometic analysis. The test was considered positive for *H. felis* when the mean of the absorbance values were two standard deviations above that of those obtained for the gastric tissues of non-infected mice. The results are shown in Table 13.

In a third experiment, two groups of C57BL/6 mice (five per group) were immunized intragastrically on days 0, 2, 14 and 16 with 100 µg rUrease plus 10 µg of the adjuvant as indicated in Table 14. A third group was immunized subcutaneously on days 0 and 16 with 10 µg rUrease plus 100 µg alum. A fourth group was immunized intragastrically on days 0, 2, 14 and 16 with 100 µg rUrease, but without adjuvant. The anti-rUrease antibody titers were determined by ELISA on pooled samples collected on day 28. Table 14 sets forth the IgA and IgG titers from sera, bronchoalveolar wash, fecal pellet extract and vaginal wash, respectively.

In a fourth experiment, five C57BL/6 mice per group were immunized as follows: Three groups of mice were immunized IG on days 0, 2, 14 and 16 with 100 µg rUrease plus 10 µg of the adjuvant as indicated in Table 15; a fourth group received no adjuvant. The anti-rUrease antibody titers were determined by ELISA on pooled samples collected on day 29. The IgA and IgG results are shown in Table 15. Table 15 also presents the IgE (PCA) and total IgE titers. PCA denotes that the IgE antibody titers were determined by the passive cutaneous anaphylaxis reaction. The PCA was performed on female Sprague-Dawley rats. The rats were sedated with ketamine/xylazine, shaved, and injected intradermally with 0.1 ml sera (serially diluted four-fold) from C57Bl/6 mice immunized with rUrease formulated with either CT, LT, or CT-CRM_{E29H}. The rats were sedated 48 to 60 hours later and then injected (0.1 ml) intravenously via the tail vein with 2 µg rUrease in PBS containing 1% Evan's blue dye.

Table 10

The effect of CT-CRMs on the generation of systemic anti-Urease IgG antibody titers in BALB/c mice

Anti-Recombinant Urease IgG Antibody Titers[a]			
Adjuvant[b]	Route[b]	Mean	SE
CT	IG	234,010	43,316
E29H	IG	131,032	64,183
R7K	IG	17,692	9,271
R11K	IG	25,502	11,413
E110D	IG	22,299	8,571
E112D	IG	8,784	5,208
CT-B	IG	47,060	38,991
QS-21	SC-R	4,038,430	1,702,556
QS-21	SC-N	5,609,764	353,824

[a] The geometric mean anti-recombinant Urease antibody titers were determined by ELISA on serum samples collected on day 28. There were 5 mice per group.

[b] The mice were immunized with 10 μg rUrease subcutaneously (SC) in the rump (R), or neck (N) on days 0 and 16. Mice immunized intragastrically (IG) received 100 μg rUrease on days 0, 2, 14 and 16. The

adjuvants were either Stimulon™ QS-21 (20 μg), CT (10 μg), or CT mutants (10 μg).

Table 11

The effect of CT-CRMs on the generation of systemic anti-Urease IgA antibody titers in BALB/c mice

Anti-Recombinant Urease IgA Antibody Titers[a]			
Adjuvant[b]	Route[b]	Mean	SE
CT	IG	2,529	584
E29H	IG	1,013	426
R7K	IG	82	15
R11K	IG	153	39
E110D	IG	351	137
E112D	IG	232	93
CT-B	IG	455	280
QS-21	SC-R	5,675	562
QS-21	SC-N	4,793	528

[a] The geometric mean anti-recombinant Urease IgA antibody titers were determined by ELISA on serum samples collected on day 28. There were 5 mice per group.

[b] The mice were immunized with 10 μg rUrease subcutaneously (SC) in the rump (R), or in the neck (N) on days 0 and 16. Mice immunized intragastrically (IG) received 100 μg rUrease on days 0, 2, 14, and 16. The adjuvants were either

Stimulon™ QS-21 (20 μg), CT (10 μg), or CT derivatives (10 μg).

Table 12

The effect of CT-CRMs on the generation of anti-Urease IgA antibody titers in the mucosal secretions of BALB/c mice

		Anti-Recombinant Urease Antibody Titers[a]			
		BAW[b]		VW[b]	
Adjuvant[c]	Route[c]	IgA	IgG	IgA	IgG
CT	IG	387	1005	3,471	464
E29H	IG	63	317	2,095	265
R7K	IG	< 5	27	79	42
R11K	IG	7	62	29	21
E110D	IG	13	98	217	84
E112D	IG	< 5	17	991	108
CT-B	IG	65	312	140	60
QS-21	SC-R	6	9816	809	10,272
QS-21	SC-N	11	10,545	235	6,237

[a] The anti-rUrease IgG and IgA antibody titers were determined by ELISA on pooled samples collected on day 29. There were 5 mice per group.

[b] BAW and VW denote bronchoalveolar and vaginal wash respectively.

[c] The mice were immunized with 10 μg rUrease subcutaneously (SC) in the rump (R), or in the neck (N) on days 0 and 16. Mice immunized intragastrically (IG) received 100 μg rUrease on days 0, 2, 14, and 16. The adjuvants were either Stimulon™ QS-21 (20 μg), CT (10 μg), or CT derivatives (10 μg).

Table 13

The generation of protective immune responses in BALB/c mice immunized with recombinant urease formulated with CT or CT-CRM_{E29H}

			IgA TITERS[a]		
Antigen[b]	Adjuvant[c]	Route	Sera	BAW[d]	No. Protected/ Total (%)[e]
PBS	CT	IG	<100	ND	2/10 (20)
r Urease	CT	IG	2,730	11	8/10 (80)
r Urease	E29H	IG	1,225	124	8/10 (80)
r Urease	QS-21	SC	14,917	7	3/10 (30)
NONE	NONE	ND	<100	ND	1/10 (10)

[a] The anti-recombinant (r) urease IgA antibody titers were determined by ELISA on pooled samples collected on day 28. There were 10 mice per group.

[b] Mice were immunized intragastrically (IG) on days 0, 2, 14 and 16 with 100 μg r urease per dose. Control mice were injected subcutaneously (SC) on days 0 and 16 with 10 μg rUrease per dose.

[c] The rUrease was formulated with either 10 μg CT or CT-CRM per dose, or mixed with 20 μg Stimulon™ QS-21 per dose.

[d] BAW denotes bronchoalveolar wash.

[e] The mice were challenged with 3 doses of 10^8 *H. felis* on days 29, 31 and 34 and assayed for protection on day 44. Protection was assessed by the rapid urease test.

Table 14

The effects of CT-CRM_{E29H} on the immune response to recombinant Urease in C57BL/6 mice

		Anti-Recombinant Urease Antibody Titers[a]							
		Sera		BAW[b]		FP[b]		VW[b]	
Adjuvant[c]	Route[c]	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
E29H	IG	1,358	252,289	14	426	265	9	40	140
CT	IG	1,117	92,182	12	331	97	7	45	309
Alum	SC	2,658	1,012,790	3	795	7	< 5	49	173
None	IG	110	10,938	< 5	14	< 5	< 5	< 50	29

[a] The endpoint anti-rUrease antibody titers were determined by ELISA on pooled serum samples collected on day 28. There were 5 mice per group.

[b] BAW, FP, and VW denote bronchoalveolar wash, fecal pellet extract, and vaginal wash respectively.

Table 14 (continued)

[c] The mice were immunized with 10 μg rUrease subcutaneously (SC) on days 0 and 16. The mice immunized intragastrically (IG) received 100 μg rUrease on days 0, 2, 14, and 16. The adjuvants were either alum (100 μg), CT (10 μg), or CT-CRM_{E29H} (10 μg).

Table 15

The generation of urease-specific IgE antibodies in the circulation of C57Bl/6 mice immunized with recombinant urease prepared with either CT, LT, or CT-CRM_{E29H}

	Anti-rUrease Antibody Titers[a]			
Adjuvant[b]	IgA	IgG	IgE (PCA)[c]	Total IgE[d]
NONE	732	30,591	<4	ND
CT	2,504	496,373	32	1591
CT-CRM_{E29H}	4,039	477,098	16	888
LT	5,251	670,807	64	3589

[a] The endpoint IgA and IgG antibody titers were determined by ELISA on pooled serum samples collected on day 29. There were 5 mice per group.

[b] The mice were immunized intragastrically (IG) with 100 μg rUrease on days 0, 2, 14, and 16. The adjuvants were 10 μg CT, CT-CRM_{E29H} or LT.

[c] PCA denotes that the IgE antibody titers were determined by the passive cutaneous anaphylaxis reaction. The PCA was performed on female Sprague Dawley rats. The rats were sedated with ketamine/xylazine, shaved, and injected intradermally with 0.1 ml sera (serially diluted 4-fold) from C57Bl/6 mice immunized with rUrease formulated with either CT, LT, or CT-CRM_{E29H}. The rats were sedated 48 to 60 hours later and then injected (0.1 ml) intravenously via the

tail vein with 2 μg rUrease in PBS containing 1% Evan's blue dye.

[d] The numbers are in ng/ml. ND denotes not detected.

Example 9

The Immune Responses of Swiss-Webster Mice Immunized with Recombinant Class 1 Pilin and Class 1 Outer Membrane Protein of *Neisseria meningitidis*

In a first experiment, 6-8 week-old Swiss-Webster mice (15 per group) were immunized IN (10 μl) at weeks 0, 2 and 3 with 5 μg of purified recombinant class 1 pilin (rpilin) formulated with CT-CRM_{E29H} (0.1 or 1 μg/mouse). Serum samples, bronchoalveolar washes (BAW), nasal washes (NW) and vaginal washes (VW) were collected from five mice in each group for determination of serum and mucosal IgA and IgG antibodies specific to *N. meningitidis* pilin by ELISA at week 4. The results are presented in Table 16. The remaining ten mice in each group immunized in parallel were challenged IN with 2 x 10^7 CFUs of the homologous *N. meningitidis* strain H355P$^+$.p2IR (passed through infant rats twice) at week 4. Recovery of Group B *N. meningitidis* from nasal tissue 24 hours post-challenge was determined by quantitative culture, as shown in Figure 2.

In a second experiment, the protection of rpilin formulated with CT-CRM_{E29H} against a homologous meningococcal strain was compared to that of CT-CRM_{E29H} admixed with an unrelated protein, KLH. Groups of five Swiss-Webster mice (six-week old) were immunized IN (10 μl volume) with 5 μg of rpilin with or without CT-CRM_{E29H} (0.1 μg), or with PorA H355 with CT-CRM_{E29H} (0.1

μg) at weeks 0, 2, and 3. Control groups were either non-immunized (naïve group) or IN immunized with KLH (5 μg) plus CT-CRM_{E29H} (0.1 μg). The endpoint antibody titers were determined by whole cell and antigen-specific ELISA on pooled serum samples collected at week 4 before challenge with 1 x 10^7 CFU's of meningococcal strain H355P$^+$. The results are presented in Tables 17 and 18.

In a third experiment, the immunogenicity of meningococcal PorA formulated with CT-CRM_{E29H} for IN immunization was assessed. Five Swiss-Webster mice per group were immunized IN on weeks 0, 2 and 3 with 20 μg/dose PorA from meningococcal strain H44/76 with or without CT-CRM_{E29H} (1 μg/dose) as indicated in Table 19. The anti-PorA H44/76 antibody titers and whole cell ELISA for IgG were assayed on pooled serum and mucosal samples collected at week 4 of the experiment. The results are presented in Table 19.

In a fourth experiment, the ability of rpilin and PorA adjuvanted with CT-CRM_{E29H} to protect mice against the challenge of a heterologous strain of meningococci was assessed. Ten Swiss-Webster mice per group were immunized IN (10 μl volume) with 5 μg of either rpilin, PorA from strain H355, or PorA from strain 870227, formulated with CT-CRM_{E29H} (0.1 μg) at weeks 0, 2, and 3. Control groups were either heat-inactivated meningococcal strain 870227 whole cells or KLH (5 μg) plus CT-CRM_{E29H} (0.1 μg). The endpoint IgG and IgA titers were determined by whole cell and antigen-specific ELISA on pooled serum samples collected at week 4 before challenge with the 870227 strain. The results are presented in Tables 20 and 21. The bacterial recovery from nasal tissue was determined by quantitative culture 24 hours after challenge with

strain 870227 and was expressed as Log_{10} CFU ± standard deviation. The results are shown in Figure 4.

A fifth experiment was conducted to examine the potential of CT-CRM_{E29H} as an adjuvant for parenteral immunization. Groups of 10 female Swiss-Webster mice, 5-6 weeks old, were immunized subcutaneously with 5 µg of PorA H355 formulated with either CT-CRM_{E29H} (10 µg) or MPL™ (100 µg) at weeks 0 and 4. Control groups were immunized subcutaneously with heat-inactivated meningococcal 870227 whole cells or KLH (5 µg) plus MPL™ (100 µg). Mice were challenged IN with 1.2×10^7 CFUs of meningococcal strain 870227 at week 6. Twenty-four hours post-challenge, mice were sacrificed and nasal tissues were homogenized and plated on selective medium. Colonies were counted after incubation at 37°C overnight and expressed as Log_{10} CFU ± standard deviation. The results of this experiment are presented in Table 22 and Figure 5.

Table 16

Adjuvant effects of CT-CRM_{E29H} on the systemic and mucosal immune responses to *N. meningitidis* rPilin (Class I H44/76) in Swiss-Webster mice

5

Immunogen	Anti-rPilin antibody ELISA titers on pooled samples									
	Sera (wk 0)		Sera (wk 4)		BW		NW		VW	
	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
rPilin	<50	<50	878	69,013	4	41	23	2	37	17
rPilin + CT-CRM_{E29H} (0.1 µg)	<50	<50	2,209	209,228	<2	19	34	40	61	51
rPilin + CT-CRM_{E29H} (1 µg)	<50	<50	4,089	1,344,776	41	540	75	45	135	216

Table 17

The effect of CT-CRM_{E29H} on the immune response to meningococcal antigens in Swiss-Webster mice

Total Sera IgG of Meningococcal B Whole Cell ELISA

Group	H355P⁺ strain		FAM 18 strain		M982 strain	
	Wk 0	Wk 4	Wk 0	Wk 4	Wk 0	Wk 4
5 µg rPilin	175*	2,371	294*	639	137*	2,375
5 µg rPilin + 0.1 µg CT-CRM_{E29H}	175*	24,965	294*	2,702	137*	21,862
5 µg PorA+ 0.1 µg CT-CRM_{E29H}	175*	10,156	294*	9,136	137*	5,733
5 µg KLH + 0.1 µg CT-CRM_{E29H}	175*	192	294*	230	137*	100

* represent week 0 pooled samples from all groups

Table 18

The effect of CT-CRM_{E29H} on the immune response to meningococcal antigens in Swiss-Webster mice

Anti-rPilin and anti-PorA antibody ELISA titers on pooled samples

Group	rPilin		Class I OMP H355	
	Sera IgG Wk 4	Sera IgA Wk 4	Sera IgG Wk 4	Sera IgA Wk 4
5 µg rPilin	8,840	<50	<50	<50
5 µg rPilin + 0.1 µg CT-CRM_{E29H}	149,221	860	120	<50
5 µg PorA H355 + 0.1 µg CT-CRM_{E29H}	<50	<50	13,795	60
5 µg KLH + 0.1 µg CT-CRM_{E29H}	<50	<50	<50	<50

Table 19

Immune responses of Swiss-Webster mice immunized intranasally with meningococcal PorA and CT-CRM_{E29H}

Anti-PorA H44/76 Antibody Titers

Group	Sera			BW		NW		VW	
		IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA
20 µg PorA	WCE	3,530	ND	ND	ND	ND	ND	ND	ND
	PorA	1,220	<50	<2	<2	<2	<2	<7	<7
20 µg PorA + 1 µg CT-CRM_{E29H}	WCE	26,660	ND	ND	ND	ND	ND	ND	ND
	PorA	17,673	<50	5	<2	<2	<2	<7	<7

ND = No data

WCE = whole cell ELISA to H44/76 strain

PorA = PorA H44/76 specific ELISA.

Table 20

Immune responses of Swiss-Webster mice immunized intranasally with heterologous and homologous meningococcal PorA and rPilin with CT-CRM_{E29H}

Assay Antigen	Serum at week	IgG titer# by ELISA in mice immunized with				
		5µg KLH + 0.1µg CT-CRM_{E29H}	5µg rPilin + 0.1µg CT-CRM_{E29H}	5µg PorA H355 + 0.1µg CT-CRM_{E29H}	5µg PorA 870227 + 0.1µg CT-CRM_{E29H}	25 µg HI WC 870227 + 0.1µg CT-CRM_{E29H}
WC 870227	0	<100	<100	<100	<100	<100
	4	350	13,376	7,088	24,815	74,930
WC H355P	0	257	257	257	257	257
	4	310	3,687	5,140	3,930	5,933
rPilin	0	146	146	146	146	146
	4	585	1,999,530	<100	<100	<100
PorA H355	0	<100	<100	<100	<100	<100
	4	<100	673	29,770	19,009	463
PorA 870227	0	<100	<100	<100	<100	<100
	4	<100	<100	10,020	23,045	5,935

Week 0 titers are pools from all groups.

WC = Whole Cell

HI = Heat-inactivated

Table 21

Immune responses of Swiss-Webster mice immunized intranasally with heterologous and homologous meningococcal PorA and rPilin with CT-CRM_{E29H}

Assay Antigen	Serum at week	IgA titer# by ELISA in mice immunized with				
		5µg KLH + 0.1µg CT-CRM_{E29H}	5µg rPilin + 0.1µg CT-CRM_{E29H}	5µg PorA H355 + 0.1µg CT-CRM_{E29H}	5µg PorA 870227 + 0.1µg CT-CRM_{E29H}	25 µg HI WC 870227 + 0.1µg CT-CRM_{E29H}
WC 870227	0	<25	<25	<25	<25	<25
	4	<25	<25	<25	<25	ND
WC H355P	0	<25	<25	<25	<25	<25
	4	<25	<25	<25	<25	<25
rPilin	0	<25	<25	<25	<25	<25
	4	<25	5,097	<25	<25	<25
PorA H355	0	<25	<25	<25	<25	<25
	4	<25	<25	233	200	<25
PorA 870227	0	<25	<25	<25	<25	<25
	4	<25	<25	<25	<25	<25

Week 0 titers are pools from all groups.

WC = Whole Cell

HI = Heat-inactivated

ND = No data

Table 22

N. meningitidis Bactericidal Activity from mouse sera subcutaneously immunized with PorA with CT-CRM_{E29H} or MPL™

Mouse sera	H355 p+	870227
wk5,d6 KLH (5µg), MPL™ (100µg)	<25	<25
wk5,d6 H355 class 1 OMP (5µg), MPL™ (100µg)	200	<25
wk5,d6 870227 class 1 OMP (5µg), MPL™ (100µg)	<25	100
wk5,d6 heat-inactivated 870227 WC (25µg), MPL™ (100µg)	25	200
wk5,d6 H355 class 1 OMP (5µg), CT-CRM_{E29H} (10µg)	<25	<25
wk0 pool negative control	<10	<10
wk6 positive control 1	200	nd
wk6 positive control 2	nd*	400

* nd = not done
Complement used: Human UR4-97

Example 10

The Immune Responses of BALB/c Mice Immunized with the Purified Native Fusion (F) Glycoprotein of Respiratory Syncytial Virus (RSV)

In a first experiment, 6-8 week old BALB/c mice (5 mice/group) were immunized intranasally (10 µl) at weeks 0 and 14 with 3 µg of purified native

protein formulated with CT-CRM_{E29H} (1 µg or 10 µg/mouse), wild-type CT (1 µg/mouse), CT-B (1 µg or 10 µg/mouse) or no adjuvant. Endpoint IgG and IgA antibody titers were assayed, by ELISA, on day 24 of the experiment. Titers were obtained from sera, bronchoalveolar lavage and vaginal washes. The results are presented in Table 23.

In a second experiment, 6-8 week old BALB/c mice (5 mice/group) were pre-immunized intranasally (50 µl) with either wild-type CT (1 µg/mouse) or CT-CRM_{E29H} (1 µg/mouse) on days 0 and 14. Control groups were not pre-immunized. Thereafter, on days 28 and 42, all mice were immunized intranasally with 3 µg of F protein formulated with the same amounts of CT or CT-CRM_{E29H}. The endpoint antibody titers were determined by ELISA on pooled samples collected on days 56 (sera) and 57 (bronchoalveolar and vaginal wash fluids). The results are presented in Table 24.

In a third experiment, naive female BALB/c mice (6-8 weeks, 5 mice/group) were immunized intranasally (IN) at weeks 0, 1 and 2 with purified native fusion (F) protein from RSV A2. Immunizations were prepared by formulating F protein (3 µg/mouse) with CT-CRM_{E29H} (1 µg or 10 µg /mouse), CT-B (1 µg or 10 µg /mouse) or alum (100 µg /mouse). The vaccine was administered intranasally by allowing anaesthetized mice to breathe in the vaccine placed at the tip of the nostril. Total volume per dose was 10 µl per mouse (in Table 25), at weeks 0, 1 and 2. Control mice received intramuscular primary immunization containing F/AlOH, or received primary and secondary immunizations of live RSV A2, delivered intranasally. Systemic humoral immune responses were assayed, by ELISA, nine

days (for Tables 25 and 26) post-tertiary immunization. Bronchoalveolar lavage, vaginal and nasal washes were also collected and utilized in the characterization of mucosal antibody responses. Spleens from immunized mice were used to assay antigen-dependent killer cell activity against MHC-compatable RSV-infected target cells. A second cohort of mice, that had received an identical immunization schedule, was challenged with live RSV. Protection of lung compartments within this cohort was subsequently analyzed at four days post-challenge by determination of virus plaques in collected homogenized lung tissue. Statistical analyses were performed using ANOVA. The results are presented in Tables 25 and 26.

In a fourth experiment, BALB/c mice were immunized intranasally at weeks 0, 1 and 2 with purified RSV F protein (3 μg/mouse) in combination with CT-CRM_{E29H} (1 μg or 10 μg/mouse), CTB (1 μg or 10 μg/mouse) or PBS. As a control, mice also received intranasal delivery of RSV or intramusular delivery of F/AlOH. Splenocytes were isolated nine days post-final immunization and stimulated *in vitro* with syngeneic RSV-infected stimulator cells. After six days in culture, antigen-dependent killer cell activity was determined by quantitation of ^{51}Chromium release by RSV-infected target cells. The results are presented in Figure 6.

In a fifth experiment, a viral protection assay, the lung compartments of immunized mice were isolated four days after challenge with live RSV, homogenized, and quantitation of infectious virus was performed. BALB/c mice were immunized intranasally with vaccines containing purified F protein from RSV and either CT-CRM_{E29H}, CTB or PBS. Groups of mice were

also immunized intramuscularly with F/AlOH as a control. Eight days after final immunization (three weeks post F/AlOH vaccine), mice were challenged with live RSV. Four days later, pulmonary tissues were harvested and utilized in the quantitation of infectious virus. The results are presented in Figure 7.

In a sixth experiment, naive female BALB/c mice (6-8 weeks, 5 mice/group) were immunized intranasally (IN) at weeks 0, 1 and 2 with purified native fusion (F) protein from RSV A2. Immunizations were prepared by formulating F protein (3 μg/mouse) with CT-CRM_{E29H} (1 μg/mouse) or alum (100 μg/mouse). The vaccine was administered intranasally by allowing anaesthetized mice to breathe in the vaccine placed at the tip of the nostril. Total volume per dose was 5 μl per mouse (in Table 27), at weeks 0, 1 and 2. Control mice received intramuscular primary immunization containing F/AlOH, or received primary and secondary immunizations of live RSV A2, delivered intranasally. Systemic humoral immune responses were assayed, by ELISA, two weeks (for Tables 27 and 28) post-tertiary immunization. Bronchoalveolar lavage, vaginal and nasal washes were also collected and utilized in the characterization of mucosal antibody responses. Spleens from immunized mice were used to assay antigen-dependent killer cell activity against MHC-compatable RSV-infected target cells. A second cohort of mice, that had received an identical immunization schedule, was challenged with live RSV. Protection of lung compartments within this cohort was subsequently analyzed at four days post-challenge by determination of virus plaques in collected homogenized lung tissue. Statistical analyses were performed using ANOVA. Results are presented in Tables 27 and 28.

In a seventh experiment, the protocol from the fourth experiment was again utilized to determine the antigen-dependent CTL activity to RSV-infected target cells. The results are presented in Figure 8.

In an eighth experiment, another viral protection assay, BALB/c mice (5 mice/group) were immunized intranasally with formulations containing purified RSV F protein, with or without CT-CRM_{E29H}. Groups of mice were also immunized intramuscularly with F/AlOH and left unimmunized (naïve) as a control. Two weeks after final immunization (four weeks post-F/AlOH administration), all groups were challenged with live RSV. Four days later, pulmonary tissues were harvested and utilized in the quantitation of infectious virus. The results are presented in Figure 9.

In a ninth experiment, a three dose protocol was employed to investigate the adjuvant response of CT-CRM_{E29H} in more detail. Groups of five BALB/c mice were immunized IN (5 µl) at weeks 0, 1 and 2 with F protein (3 µg), admixed with either 0.01, 0.1 or 1.0 µg CT-CRM_{E29H}. Control mice were primed at day 0 with F/AlOH (intramuscular) or RSV A2 (IN). Serum antibody titers were determined two weeks post-tertiary immunization. The results are presented in Table 29. Data are presented as the $\log_{10}$ geometric mean antibody titer (± 1 SD). Similar results were obtained in two separate studies.

After IN immunization with F/CT-CRM_{E29H}, the mice in the ninth experiment were also tested for their local antibody responses to F protein. Mucosal wash samples were taken from mice sacrificed two weeks post-tertiary immunization and analyzed for anti-F protein-specific IgG and IgA by ELISA. The results are presented in Table 30. Data are presented as the $\log_{10}$

of the geometric mean endpoint titer that resulted in an OD_{410} of 0.03. Similar results were obtained in two separate studies.

In a tenth experiment, in order to investigate the functional capacity of the humoral immune responses induced by F/CT-CRM_{E29H} immunization, sera were tested in a plaque reduction assay for neutralizing antibody titers to RSV A2. The results are presented in Table 31. Geometric mean neutralizing antibody titers (log_{10}) were determined on individual sera (five mice per group) in the presence (+C') and absence (-C') of 5% guinea pig serum as a complement source. Similar results were obtained in two separate studies.

In an eleventh experiment, mice (five mice per group) received IN immunizations of F protein formulated with 0.01, 0.1 or 1 μg of CT-CRM_{E29H} on days 0, 7 and 14. Control mice were immunized with F/AlOH (intramuscular) or RSV A2 (IN). Immunized mice were challenged two weeks after tertiary immunization, in order to determine the ability of F/CT-CRM_{E29H} to protect against subsequent infection. Four days post-infection, virus levels were determined in homogenized lung and nose tissues of individual mice. The results are presented in Table 32. Data are presented as the geometric mean virus titer per g of tissue (± 1 standard deviation).

Table 23

The generation of anti-cholera toxin antibodies in BALB/c mice after intranasal immunization with either CT, CT-B, or CT-CRM_{E29H}

Anti-Cholera Toxin Antibody Titers[a]

	Sera		BAW		VW	
Adjuvant[b]	IgG	IgA	IgG	IgA	IgG	IgA
None	<100	<100	<25	<25	<25	<25
CT (1 μg)	434,180	3,944	70	<25	214	1,431
CT-B (1 μg)	781,628	4,665	280	<25	127	709
CT-B (10 μg)	156,239	2,544	241	<25	163	1,243
E29H (1 μg)	118,541	2,207	94	<25	227	1,578
E29H (10 μg)	514,233	4,881	410	ND[c]	214	980

[a] The endpoint antibody titers were determined on pooled samples collected on day 24 of the study. BAW and VW denote bronchoalveolar and vaginal wash respectively. There were 5 mice per group.

Table 23 (continued)

[b] The mice were immunized intranasally (10 µl) on days 0 and 14 with 3 µg of native F protein admixed with the indicated amount of either wild-type CT, commercial CT-B, or CT-CRM_{E29H}.

[c] ND = No data

Table 24

The effect of pre-existing anti-cholera toxin antibodies on the immunogenicity of the fusion protein of respiratory syncytial virus formulated with either CT or CT-CRM_{E29H}

Anti-Fusion Protein Antibody Titers[a]

		Sera		BAW		VW	
Pre-Vax[b]	Adjuvant	IgG	IgA	IgG	IgA	IgG	IgA
None	None	<1,000	<100	<25	<25	<25	25
None	CT	887,136	11,337	16,219	8,709	274	1,447
+	CT	>10,000,000	22,344	253,641	24,331	36,415	4,391
None	E29H	1,430,836	10,786	2,232	1,680	333	378
+	E29H	6,346,730	17,890	131,217	12,367	2,750	6,359

[a] The endpoint antibody titers were determined by ELISA on pooled samples collected on days 56 (sera) and 57 (bronchoalveolar (BAW) and vaginal (VW) wash fluids). There were 5 mice per group.

[b] The mice were pre-immunized (intranasally, 50 µl) with either wild-type CT (1 µg) or CT- CRM_{E29H} (1 µg) on days 0 and 14. Thereafter on days 28 and 42 all mice were immunized (intranasally, 50 µl) with 3 µg of F protein formulated with same amounts

Table 24 (continued)

(1 μg) of either wild-type CT or CT-CRM_{E29H}. The endpoint anti-CT antibody titers of mice pre-immunized with either wild-type CT or CT-CRM_{E29H} and subsequently immunized with F protein were greater than 1,000,000 on day 42.

Table 25

Systemic Immune Responses of BALB/c mice immunized intranasally with RSV F protein and CT-CRM_{E29H}

		Anti-F Antibody Titers			
Group	Immunogen	IgG	IgG1	IgG2a	IgA
776	10 µg E29H	<100	<100	<100	<100
777	3 µg F 1 µg E29H	126,463 +/- 32,646	62,344 +/- 27,002	4,899 +/- 1,027	16,202 +/- 2,031
778	3 µg F 10 µg E29H	209,123 +/- 75,688	75,711 +/- 29,659	19,425 +/- 13,508	15,706 +/- 10,909
779	3 µg F 1 µg CTB	46,742 +/- 32,987	26,902 +/- 14,985	4,239 +/- 3,658	4,076 +/- 614
780	3 µg F 10 µg CTB	285,116 +/- 110,154	116,245 +/- 34,596	10,512 +/- 11,016	11,679 +/- 7,246
784	3 µg F PBS	2,171 +/- 1,921	521 +/- 743	<100	<100
785	3 µg F 100 µg AlOH	23,303 +/- 16,994	5,519 +/- 2,348	<1000	<100
907	RSV A2	52,749 +/- 23,557	6,252 +/- 4,286	8,718 +/- 2,826	4,284 +/- 2,350

For total IgG:

p<0.05: 777 to 780 versus 784; 780 vs 779; 777 to 780 vs 785; 777, 778, 780 vs 907 (779 vs 907 p=0.7)

p>0.05: 778 vs 777 (p=0.125);

For IgG1:

p<0.05: 777 to 780 vs 784; 777 to 780 vs 907; 777 to 780 vs 785

p>0.05: 781 to 780 vs 907

For IgG2a

p<0.05: 777 to 780 vs 784

Table 26

Mucosal Immune Responses of BALB/c mice immunized intranasally with RSV F protein and CT-CRM_{E29H}

Group	Immunogen	Anti-F Antibody Titers					
		BAL pools		VW pools		NW pools	
		IgG	IgA	IgG	IgA	IgG	IgA
776	10 µg E29H	<25	<25	<25	<25	<25	<25
777	3 µg F + 1 µg E29H	463	<25	253	2855	<25	237
778	3 µg F + 10 µg E29H	370	<25	239	848	491	242
779	3 µg F + 1 µg CTB	137	<25	298	426	77	272
780	3 µg F + 10 µg CTB	1109	226	903	3574	512	372
784	3 µg F PBS	<25	<25	<25	78	<25	<25
785	3 µg F + 100 µg AlOH	<25	<25	<25	<25	<25	<25
907	RSV A2	2870	1126	167	738	172	170

Table 27

Systemic Immune Responses of BALB/c mice immunized intranasally with RSV F protein and CT-CRM$_{E29H}$

		Anti-F Antibody Titers			
Group	Immunogen	IgG	IgG1	IgG2a	IgA
250	3 µg F PBS	<100	<100	<100	<100
256	3 µg F 1 µg E29H	315,878 +/- 131,746	78,380 +/- 40,870	10,718 +/- 16,475	444 +/- 1,458
257	1 µg E29H	<100	<100	<100	<100
258	3 µg F 100 µg AlOH	121,551 +/- 52,023	63,595 +/- 27,491	428 +/- 4,205	<100
259	RSV A2	112,451 +/- 50,247	8,871 +/- 5,206	9,953 +/- 4,924	224 +/- 344

For total IgG:
p<0.05: 256 vs 250 and 257
p>0.05: 256 vs 258 and 259
For IgG1:
p<0.05: 256 vs 250 and 257
p>0.05: 256 vs 258
For IgG2a
p<0.05: 256 vs 250, 257 and 258
p>0.05: 256 vs 259
For IgGA
p>0.05: 256 vs 259

Table 28

Mucosal Immune Responses of BALB/c mice immunized intranasally with RSV F protein and CT-CRM_{E29H}

Group	Immunogen	Anti-F Antibody Titers					
		BAL pools		VW pools		NW pools	
		IgG	IgA	IgG	IgA	IgG	IgA
250	3 µg F PBS	<25	<25	<25	<25	<25	<25
256	3 µg F 1 µg E29H	826	<25	1,195	3,730	554	875
257	1 µg E29H	<25	<25	<25	<25	<25	<25
258	3 µg F 100 µg AlOH	706	<25	577	108	148	<25
259	RSV A2	347	<25	172	1,449	305	<25

Table 29

Anti-F Protein Serum Antibody Response Induced After Immunization with F Protein and CT-CRM_{E29H}

	Geometric mean antibody titers (log_{10})						
	Anti-F protein					Anti-CT	
Immunogen	IgG	IgG1	IgG2a	IgG2b	IgA	IgG	IgA
F/PBS	3.0 ± 1.0	2.9 ± 0.9	2.2 ± 0.4	2.4 ± 0.6	<2.0	<3.7	<2.0
CT-CRM_{E29H} (1.0 µg)	<2.0	<2.0	<2.0	<2.0	<2.0	6.4 ± 0.3	4.4 ± 0.1
F/CT-CRM_{E29H} (1.0 µg)	5.6 ± 0.3[a,b]	5.2 ± 0.3[a,b]	4.5 ± 0.3[a,b]	5.0 ± 0.3[a,b]	3.0 ± 0.6[a,b]	6.4 ± 0.2[c]	4.3 ± 0.2[c]
F/CT-CRM_{E29H} (0.1 µg)	5.4 ± 0.2[a]	5.1 ± 0.2[a]	3.9 ± 0.7[a]	4.6 ± 0.5[a]	3.1 ± 0.2[a]	5.5 ± 0.2[d]	3.8 ± 0.2[d]
F/CT-CRM_{E29H} (0.01 µg)	3.8 ± 0.8[e]	3.5 ± 0.9[e]	2.3 ± 0.7[e]	3.0 ± 0.7[e]	2.1 ± 0.2[e]	<3.7	<2.0
F/AlOH	4.9 ± 0.8	4.7 ± 0.7	3.5 ± 0.9	3.7 ± 1.0	<2.0	ND[g]	ND
RSV A2	4.9 ± 0.1	4.0 ± 0.2	4.4 ± 0.2	4.1 ± 0.3	2.3 ± 0.3[f]	ND	ND

[a] $p < 0.05$ compared to F/PBS or F/CT-E29H (0.01 µg),

[b] $p > 0.05$ compared to F/CT-E29H (0.1 µg) or F/AlOH.

[c] $p < 0.05$ compared to F/CT-E29H (0.1 and 0.01 µg) and F/PBS. $p > 0.05$ compared to CT-E29H (1.0 µg).

[d] $p < 0.05$ compared to F/PBS and F/CT-E29H (0.01 µg).

Table 29 (continued)

[e]p > 0.05 compared to F/PBS.

[f]p < 0.05 compared to F/ CT-E29H (0.1 or 1.0 μg).

[g]ND = not done.

Table 30

Anti-F Protein Antibodies in the Mucosal Fluids of Mice Immunized with F Protein Formulated with CT-CRM_{E29H}

Immunogen	BAL		VW		NW	
	IgG	IgA	IgG	IgA	IgG	IgA
F/PBS	<1.4	<1.4	<1.4	<1.4	<1.4	<1.4
CT-CRM_{E29H} (1 μg)	<1.4	<1.4	<1.4	<1.4	<1.4	<1.4
F/CT-CRM_{E29H} (1 μg)	3.1	<1.4	2.8	3.5	2.0	2.2
F/CT-CRM_{E29H} (0.1 μg)	2.5	<1.4	2.5	2.9	<1.4	2.3
F/CT-CRM_{E29H} (0.01 μg)	<1.4	<1.4	<1.4	2.2	<1.4	<1.4
F/AlOH	2.4	<1.4	<1.4	<1.4	<1.4	<1.4

Table 31

Generation of Systemic Anti-RSV Neutralizing Antibodies After IN Immunization with F Protein Formulated with CT-CRM_{E29H}

Immunogen	Neutralizing antibody titer(+C')	Neutralizing antibody titer(-C')
F/PBS	<1.3	<1.3
CT-CRM_{E29H} (1 μg)	<1.3	<1.3
F/CT-CRM_{E29H} (1 μg)	2.3 ± 0.7[a]	<1.3
F/CT-CRM_{E29H} (0.1 μg)	2.2 ± 0.4[b]	<1.3
F/CT-CRM_{E29H} (0.01 μg)	<1.3	<1.3
F/AlOH	2.0 ± 0.3	<1.3
RSV A2	2.3 ± 0.3	<1.3

[a] $p < 0.05$ compared to F/PBS or F/CT-CRM_{E29H} (0.01 μg), $p > 0.05$ compared to F/CT-CRM_{E29H} (0.1 μg), F/AlOH or RSV A2.

[b] $p < 0.05$ compared to F/PBS or F/CT-CRM_{E29H} (0.01 μg), $p > 0.05$ compared to F/CT-CRM_{E29H} (1 μg), F/AlOH or RSV A2.

Table 32
Virus Infectivity of Lung and Nasal Tissue After IN Immunization with F Protein and CT-CRM_{E29H}

Immunogen	Lung virus titer (log_{10} mean ± SD)	Nasal virus titer (log_{10} mean ± SD)
F/PBS	4.6 ± 0.5	2.7 ± 0.2
CT-CRM_{E29H} (1 μg)	4.6 ± 0.5	3.5 ± 0.2
F/CT-CRM_{E29H} (1 μg)	<2.0 ± 0.1[a,b]	<1.9 ± 0.1 [c]
F/CT-CRM_{E29H} (0.1 μg)	<1.9 ± 0.1 [a]	<1.8 ±0.1 [d]
F/CT-CRM_{E29H} (0.01 μg)	3.9 ± 0.7	2.9 ± 0.4
F/AlOH	2.6 ± 0.7	2.3 ± 0.4
RSV	<2.0 ± 0.03	<1.8 ± 0.1
Naive	4.6 ± 0.1	3.4 ± 0.5

[a] $p < 0.05$ compared to F/PBS, F/CT-CRM_{E29H} (0.01 μg), CT-CRM_{E29H} or naive, $p > 0.05$ compared to F/AlOH or RSV A2.

[b] $p > 0.05$ compared to F/CT-CRM_{E29H} (0.1 μg).

[c] $p < 0.05$ compared to F/CT-CRM_{E29H} (0.01 μg), F/PBS, CT-CRM_{E29H} or naive, $p > 0.05$ compared to F/CT-CRM_{E29H} (0.1 μg), F/AlOH or RSV A2.

[d] $p < 0.05$ compared to F/PBS, F/CT-CRM_{E29H} (0.01 μg), CT-CRM_{E29H} or naive, $p > 0.05$ compared to F/CT-CRM_{E29H} (1

μg), F/AlOH or RSV A2.

Example 11

The Immune Responses of Mice Immunized with Rotavirus Recombinant Virus-Like Particles

Spodoptera frugiperda (Sf-9) cells (American Type Culture Collection, Manassas, VA) were maintained in SF-900 II serum free medium (Gibco-BRL, Grand Island, NY). Sf9 cells were co-infected with recombinant baculovirus constructs expressing VP2 and VP6 genes from Simian rotavirus strain SA11 (32).

Released 2/6-VLPs were purified from the growth medium of these infected Sf9 cells as follows. The cells were clarified by centrifugation at 830 x g for 30 minutes at room temperature. The supernatants were then further clarified by centrifugation at 8000 x g for 30 minutes. VLPs were purified from the supernatants by centrifugation twice through 35% sucrose in TNC buffer (10 mM Tris, 140 mM NaCl, 10 mM $CaCl_2$, pH 8.0) at 96500 x g for two hours, then suspended in TNC buffer and stored at 4°C. Purified VLPs were analyzed by SDS-PAGE, followed by silver and Commassie brilliant blue staining to determine purity, Western blot analysis to analyze protein composition, electron microscopy to determine integrity of the particles, and BCA protein assay to measure total protein concentration.

Commassie brilliant blue, silver staining and Western blot analysis of purified 2/6-VLP confirmed the presence of VP2 and VP6 proteins, as well as their purity and immunoreactivity with specific monoclonal antibodies. Purity of VLPs was estimated to be about 95% from the band intensities on the gels. In addition, electron microscopic analysis of these

purified 2/6-VLPs confirmed their morphological integrity (data not shown).

Mice were immunized as follows. BALB/c and CD-1 mice used in this study were purchased from Charles River Laboratories (Storeridge, NY), bred in a rotavirus-free environment. Four week old BALB/c mice were immunized twice on week 0 and 2, either orally (n=4) or IN (n=5, n=4), with 100 and 10 μg of 2/6-VLPs respectively; each dose was formulated with 10 μg of CT-CRM_{E29H}. A third group of BALB/c mice (n=4) received 2/6-VLPs with CT-CRM_{E29H} IN, followed by an oral booster immunization (i.e., mixed group). Control mice in this experiment were immunized with CT-CRM_{E29H} (n=10), 1X TNC buffer (n=5) or 2/6-VLPs plus 1X TNC buffer (n=5). Each mouse was immunized IN with 20 μl of inoculum, 2 μl at a time, into alternating nares at one minute intervals. Serum and fecal samples were collected from all animals on weeks 0, 1, 2, 4, 6, 8, 10, 13 and the levels of rotavirus-specific serum IgG, IgM and IgA antibodies produced, as well as fecal IgG and IgA, were determined by ELISA. The serum antibody results are presented in Figures 10 and 11; the fecal antibody results are presented in Figure 13. Week 13 serum samples were used to determine IgG1 and IgG2a subclasses. The antibody subclass results are presented in Figure 12.

Pre-immunization sera diluted 1:100 and 1:2 dilutions of pre-immunization stool samples showed no reactivity in ELISA. Sera and stools from controls receiving only TNC buffer or CT-CRM_{E29H} were analyzed in parallel. The control groups showed no rotavirus-specific serum or fecal antibodies throughout the study.

Four week old CD-1 mice were immunized three

times orally (n=4) or IN (n=4) on weeks 0, 2 and 13 as above using CT-CRM_{E29H} as the adjuvant. A control group (n=2) received CT-CRM_{E29H} alone. Serum and stool samples were collected on weeks 0-9, 11-14, 26-28 and the levels of rotavirus-specific serum and fecal antibodies were determined.

For detection and quantification of IgG, IgM and IgA in stool and serum samples, 96-well polyvinyl chloride microtiter plates (Dynex Technologies, Chantilly, VA) were coated with a hyperimmune guinea pig anti-SA11 rotavirus serum diluted in phosphate buffered saline (PBS) and incubated at 37°C for four hours, or overnight at room temperature. The plates were then blocked with 5% BLOTTO (5% w/v nonfat powdered milk in PBS) at 37°C for two hours. Suspended stool samples were diluted 1:1 in 1% BLOTTO and added to the plates. The plates were then incubated overnight at 4°C, after which they were washed three times with TNC buffer plus 0.05% Tween™ 20 (TNC-T). Rabbit anti-rhesus rotavirus hyperimmune serum was diluted in 1% BLOTTO plus 2.5% normal guinea pig serum (NGPS) and added to the plates for one hour at 37°C. The plates were then washed three times with TNC-T. Horseradish peroxidase-conjugated goat anti-rabbit IgG, IgM and IgA (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was diluted in 1% BLOTTO plus 2.5% NGPS and added to the plates, which were incubated for one hour at 37°C. The plates were then washed four times with TNC-T. TMB substrate (Kirkegaard and Perry Laboratories) was added, and the color reactions produced were allowed to develop for seven minutes at room temperature. The reaction was stopped by the addition of 1M phosphoric acid. The OD was determined at 450 nm using a micro-plate reader (BIO-TEK Instrument, Winooski, VT). Measurements of 0.1 above

the blank were considered significant. SA11 stock virus was diluted in 1% BLOTTO and added to the plates, which were then incubated overnight at 4°C. The plates were washed three times with TNC-T; thereafter, stool samples diluted 1:1 in 1% BLOTTO or serum samples diluted serially in 1% BLOTTO were applied. As a negative control, duplicates of stool samples were added to a well with no anti-SA11 antibody coating. The plates were incubated for two hours at 37°C and then washed three times with TNC-T. Peroxidase-conjugated goat anti-mouse IgG, IgM and IgA were diluted in 1% BLOTTO plus 2.5% NGPS and added to wells; peroxidase-conjugated goat anti-mouse IgA+IgG+IgM(H+L) antibodies were similarly diluted and added to wells for pre-immunization antibody detection. The plates were incubated for one hour at 37°C and then washed four times with TNC-T. The plates were developed as described above for the antigen detection ELISA. The ELISA protocol used to determine IgG subclasses was a modification of the protocol described that employed HRP-labeled rat (monoclonal) anti-mouse IgG1 and IgG2a as the secondary antibodies (Biosource International, Camarillo, CA).

An immunohistochemical assay, described by Ishida et al (49), was modified and used to detect anti-rotaviral VP2 and VP6 antibodies in the serum of mice immunized with 2/6-VLPs. Briefly, early log phase Sf9 cells in shaker flasks were seeded into 96 well tissue culture plates at a density of 2.5×10^4 cell/well and then incubated one hour at room temperature (RT). Subsequently, cells were infected with recombinant baculoviruses encoding VP2 or VP6 genes at a multiplicity of infection of 10, and the infection was allowed to proceed at 28°C for three days. The culture medium was then discarded, plates

were dried in a vacuum oven at RT for one hour and fixed with 10% formalin (37% formaldehyde solution containing 10-15% methanol; Sigma) in PBS at RT for 30 minutes. Cells were subsequently permeablized with 1% Triton X-100 (Sigma) in TNC buffer at RT for five minutes.

Each set of infected cells expressing the designated rotavirus protein was exposed to pre-challenge or post-immunization serum from each BALB/c or CD-1 mouse, followed by immunostaining. Mouse serum samples were serially diluted in PBS with 5% FCS. Samples were added to the wells and the plates were incubated at 37°C for two hours. Plates were then washed four times with PBS. Horseradish peroxidase labeled goat anti-mouse IgG, IgM or IgA antibody (Kirkegaard & Perry Laboratories) was added in PBS with 5% FCS, and incubated at 37°C for one hour. Stained cells were detected with 3-amino-9-ethyl-carbazole substrate (AEC)(Sigma) after washing the wells twice with PBS. Uninfected Sf9 cells, serum from unimmunized mice, and pre-immunization sera from immunized mice were used as negative controls. Monoclonal antibodies against VP6 (7D9, 5E6) and VP2 (BP2) were used as positive controls (50).

Unimmunized animals and those immunized with 2/6-VLPs were challenged by gavage with 10 SD_{50} of wild-type murine EDIM rotavirus (51) on week 26 (CD-1 mice) or on week 13 (BALB/c mice). The titer of EDIM strain was determined as shedding dose 50 (SD_{50}), the dose required to induce fecal viral shedding in 50% of adult mice. The trypsin-activated challenge virus (100 μl) was administered following oral administration of 100 μl of 4% sodium bicarbonate solution to neutralize gastric acidity. Viruses were diluted in M199 media (Irvine Scientific, Santa Ana, CA) and activated with

10 μl of trypsin stock (1 mg/ml) (Sigma Chemical Co, St. Louis, MO) per ml of viral stock solution. Following challenge, stool samples were collected from all animals for 9 days.

Rotavirus antigen shedding in fecal samples was measured by ELISA and expressed as net optical density (OD) values, i.e., OD of the post-challenge fecal sample minus OD of the pre-challenge sample from the same mice. The area under the shedding curve for each animal was determined and the percent reduction in antigen shedding (PRAS) for each animal was calculated by comparing the area under the curve for each animal to the mean area of the control group. The mean PRAS was then calculated for each immunized group. Only PRAS levels above 50% were considered protective. The results are presented in Figure 14.

Statistical analyses were performed with SPSS for version 8.0 for Windows (SPSS, Inc., Chicago, IL.). Independent t tests were used to compare pre-challenge geometric mean titer values and to compare PRAS between groups. Up to three digits after the decimal point were considered in calculating P values (0.000=0).

Example 12

Increasing the Expression of CT-CRM$_{E29H}$ Through Use of an Arabinose Inducible Promoter

The construction of the arabinose inducible system was as follows: PCR primers were synthesized to amplify the toxin encoding bicistron from pIIB29H. Primer #1, CT29 Forward *Nhe*I: 5'-TTTTTTGGGCTAGCATGGAGGAAAAGATG AGC-3' (SEQ ID NO:6); Primer #2, CT29 Reverse *Hind*III: 5'-GCAGGTCGAAGCTTGCATGTTTGGGC-3' (SEQ ID NO:7). The CT-

CRM_{E29H} *ctxA* and *ctxB* encoding PCR fragment was cloned into pBAD18-Cm using the endonuclease sites *Nhe*I and *Hind*III, resulting in construct pCT18. The *ctxB* (*V. cholerae* 2125) gene from pCT18 was removed using *Cla*I and *Hind*III and replaced with a similar fragment from plasmid pMGJ142 which was shown to encode a *ctxB* gene from *V. cholerae* strain 569B. The resulting construct, pPX7490 encodes the CT-CRM_{E29H} *ctxA* and *ctxB* genes from strain 569B under control of the arabinose promoter, and has the LTIIb-B leader sequence.

Protocols for the large scale expression and purification of CT-CRM_{E29H} were developed and are as follows: 3L fluted Fernbach flasks were used, with 1L per flask of Hy-Soy growth media (per liter: Hy-Soy 10 g; yeast extract 12.5 g; sodium chloride 5 g; sodium phosphate, monobasic 3.3 g; sodium phosphate, dibasic 13.1 g). For starter-stock preparation, 20 µg/ml chloramphenicol and 20 ml of sterile 50% glucose were added to one flask (1% final glucose concentration). The flask was then inoculated with 300 µl DH5α transformed with pPX7490, -70°C frozen stock. The flask was incubated at 37°C with shaking at 200 rpm overnight. All growth media to be used the following day was prewarmed by shaking overnight at 37°C at 200 rpm. The following day, a 1:40 dilution of the overnight culture was made into Hy-Soy growth media, supplemented with 20µg/ml chloramphenicol and 10 ml sterile glycerol (1% final glycerol concentration) as a carbon source. This culture was outgrown at 37°C to an OD_{600} of 4.5-5.5 in Fernbach flasks (or higher in a bioreactor). The culture was then induced with 20 ml L-Arabinose (0.5% final concentration) and allowed to incubate for an additional three hours. After a three hour induction period, the majority of toxin was cell-

associated. Cells were harvested by centrifugation and the pellet resuspended into 10 mM $NaPO_4$, 1 mM EDTA (pH 7.0) buffer at 9% of original culture volume. The cell suspension was mechanically disrupted through a microfluidizer and centrifuged for ten minutes at 8500xg to remove cellular debris. The cellular lysate (supernatant) was further clarified at 42,000 rpm for one hour in a 45Ti rotor, 160,000xg $_{AV}$. The clarified cell lysate was loaded onto a carboxymethyl(CM)-Sepharose™ column (Pharmacia) equilibrated with 10mM $NaPO_4$ (pH 7.0). Approximately 300 ml CM-Sepharose™ was used per 10 liters of culture volume. The cell lysate was loaded onto the column at the rate of 0.102 cm/min (2 ml/min). The column was washed extensively with 10-15 column volumes of 10 mM $NaPO_4$ (pH 7.0) at 0.255 cm/min (5 ml/min) to remove contaminants. The CT-CRM_{E29H} holotoxin was eluted with four column volumes of 10mM $NaPO_4$ (pH 8.3) at 0.255 cm/min. The CT-CRM_{E29H}-containing eluate was buffer exchanged by filtration or dialyzed against PBS, then stored at 4°C.

Example 13

The Immune Responses of BALB/c Mice Immunized with Plasmid DNA Encoding the Full Length Glycoprotein D of Herpes Simplex Virus Type 2

Six to eight week old female BALB/c mice were immunized with plasmid DNA (pDNA) encoding the full length glycoprotein D of herpes simplex virus type 2 (gD2) (the plasmid containing the gD2 gene is described in Pachuk et al. (40), which is hereby incorporated by reference), formulated with 0.25% bupivacaine, and adjuvanted with wild-type CT, CT-CRM_{E29H} or no adjuvant. Animals were given a secondary immunization three weeks

following the primary and euthanised two weeks after the last injection. The protocol for immunization was as follows.

Table 33

Group	Route of Delivery	Conc. of Plasmid	Conc. of Adjuvant	Volume Injected
Mouse 1	ID	50 µg 024	50 µg CT-CRM_{E29H}	10 µl
Mouse 2	ID	50 µg 024	50 µg CT	10 µl
Mouse 3	ID	50 µg 024	-	10 µl
Mouse 4	IM	50 µg 024	-	100 µl
Mouse 5	ID	50 µg 023	-	10 µl

024 - pDNA including gD2 gene; 023 = pDNA backbone without gD2 gene inserted
ID = Intradermal; IM = Intramuscular
Group 4 served as a positive control; Group 5 served as a negative control.

A proliferation assay was conducted as follows: $1X10^5$ spleen cells were cultured in the presence or absence of 200ng/ml of gD2 protein in complete RPMI media with 10% FCS. After four days incubation at 37°C in the presence of 5% CO_2, cultures were pulsed overnight with ^{3}[H]. ^{3}[H] incorporation was measured on a beta counter. The counts were reported as SI (Stimulation Index = counts in presence of antigen stimulation divided by counts in absence of antigen stimulation). The results are presented in Table 34.

An ELISA was carried out to measure the antigen-specific humoral response in sera and vaginal

washes. Briefly, 96 well flat bottom plates (Maxisorb, Nunc) were coated overnight at 4°C with purified gD2 protein at a concentration of 0.4 µgs/ml. The plates were washed three times with PBS and blocked with 4% BSA for one hour at room temperature. Fifty microliters (1:100 dilution) of serum samples or 50µl of vaginal wash sample was added to the plate. After incubation for one hour for sera and overnight at 4°C for vaginal wash, the plates were washed with PBS five times, and a 1:3000 dilution of peroxidase-conjugated anti-mouse Ig (Sigma, St. Louis, MO) was added and the plates incubated for one hour. The plates were washed with PBS before adding the substrate 3,3',5,5'-tetramethylbenzidine (TMB)-H_2O_2 (Biotecx, Houston, TX). Color was allowed to develop for 30 minutes before reading at 450 nm on a E_{max} microplate reader (Molecular Devices, Sunnyvale, CA). The results are presented in Table 35 (sera) and Table 36 (vaginal washes).

Cytokines were measured using a standard ELISA as described above. Plates were suitably coated to capture either IL-5 or gamma interferon from supernatants from 24 hour or 72 hour old gD2-stimulated cultures respectively. The results are presented in Table 37.

Table 34
gD2-Specific Cellular Proliferation Response (SI) After Administration of pDNA for HSV gD2 with CT or CT-CRM_{E29H}

Group	ID CT	ID CT-CRM_{E29H}	ID	IM
Mouse 1	6521	28826	24696	30949
Mouse 2	9641	15760	20249	26159
Mouse 3	32078	25558	12472	35366
Mouse 4	28792	19023	7092	5151
Mouse 5	12486	22510	14702	30790
Average	17904	22335	15842	25683

Table 35
gD2-Specific Humoral Response (ng/ml) After Administration of pDNA HSV gD2 with CT or CT-CRM_{E29H} in Sera

Group	ID CT	ID CT-CRM_{E29H}	ID	IM
Mouse 1	-27	606	516	932
Mouse 2	-15	1387	1547	1315
Mouse 3	333	33	113	430
Mouse 4	582	755	688	108
Mouse 5	3	208	13	234
Average	175	598	575	604

Table 36

gD2-Specific Humoral Response (ng/ml) After Administration of pDNA HSV gD2 with CT or CT-CRM_{E29H} in Vaginal Washes

Group	ID CT	ID CT-CRM_{E29H}	ID	IM
Mouse 1	0	0	69	374
Mouse 2	0	224	0	198
Mouse 3	0	145	0	83
Mouse 4	0	0	347	0
Mouse 5	0	-49	0	0
Average	0	70	54	112

Table 37

gD2-Specific Cytokine ELISA Profile (pg/ml)

	024+ CT ID	024+ CT-CRM_{E29H} ID	024 ID	024 IM	023 Con-trol
gamma IFN	1597	1751	136	716	505
IL-5	63	209	9	388	16

Bibliography

1. Mekalanos, J.J., et al., Nature, 306, 551-557 (1983).

2. Gill, D.M., Biochemistry, 15, 1242-1248 (1976).

3. Cuatrecasas, P., Biochemistry, 12, 3558-3566 (1973).

4. Kassis, S., et al., J. Biol. Chem., 257, 12148-12152 (1982).

5. Mekalanos, J.J., et al., J. Biol. Chem., 254, 5855-5861 (1979).

6. Gill, D.M., and Meren, R., Proc. Natl. Acad. Sci., USA, 75, 3050-3054 (1978).

7. Welsh, C.F., et al., "ADP-Ribosylation Factors: A Family of Guanine Nucleotide-Binding Proteins that Activate Cholera Toxin and Regulate Vesicular Transport", pages 257-280 in Handbook of Natural Toxins: Bacterial Toxins and Virulence Factors in Disease Vol. 8 (Moss, J., et al., Eds., Marcel Dekker, Inc., New York, NY (1995)).

8. Hanson, L.A., Intl. Arch. Allergy Appl. Immunol., 18, 241-267 (1961).

9. Tomasi, T.B., and Zigelbaum, S., J. Clin. Invest., 42, 1552-1560 (1963).

10. Tomasi, T.B., et al., J. Exptl. Med., 121, 101-124 (1965).

11. Brandtzaeg, P., and Baklein, K., Scand. J. Gastroenterol., 11 (Suppl. 36), 1-45 (1976).

12. Brandtzaeg, P., "Immune Functions of Human Nasal Mucosa and Tonsils in Health and Disease", page 28 et seq. in Immunology of the Lung and Upper

Respiratory Tract (Bienenstock, J., Ed., McGraw-Hill, New York, NY (1984)).

13. Solari, R., and Kraehenbuhl, J-P, Immunol. Today, 6, 17-20 (1985).

14. Crabbe, P.A., et al., J. Exptl. Med., 130, 723-744 (1969).

15. Bazin, H., et al., J. Immunol., 105, 1049-1051 (1970).

16. Craig, S.W., and Cebra, J.J., J. Exptl. Med., 134, 188-200 (1971).

17. Mestecky, J., et al., J. Clin. Invest., 61, 731-737 (1978).

18. Elson, C.O., and Ealding, W., J. Immunol., 132, 2736-2741 (1984).

19. Maneval, D.R., et al., J. Tissue Cult. Methods, 6, 85-90 (1981).

20. Guidry, J.J., et al., Infect. Immun., 65, 4943-4950 (1997).

21. United States Patent number 5,601,831.

22. United States Patent number 5,108,744.

23. International Patent Application number WO 96/05858.

24. Hu, L.T., et al., Infect. Immun., 60, 2657-2666 (1992).

25. International Patent Application number PCT/US99/09486, filed April 29, 1999.

26. European Patent Application number 449,958.

27. Walsh, E.E., et al., Infect. Immun., 43, 756-758 (1984).

28. Crawford, S.E., et al., J. Virology, 68, 5945-5952 (1994).

29. Lee, A.J., et al., Gastroenterology, 112, 1386-1397 (1997).

30. Snider, D.P., et al., J. Immunol. 153, 647-657 (1994).

31. Tamura, S.Y., et al., Vaccine, 12, 1238-1240 (1994).

32. Van der Akker, F., et al., Structure, 4, 665-678 (1996).

33. Rudin A., et al., Infect. Immun., 66, 3390-3396 (1998).

34. O'Neal, C.M., et al., J. Virology, 71, 8707-8717 (1997).

35. O'Neal C.M., et al., J. Virology, 72, 3390-3393 (1998).

36. International Patent Application number WO 93/13202.

37. International Patent Application number WO 98/42375.

38. International Patent Application number WO 98/20734.

39. United States Patent number 5,593,972.

40. Pachuk, C., et al., Curr. Topics Microbiol. Immunol., 59, 1024-1031 (1997).

41. Jobling, M.G., and Holmes, R.K., Infect. Immun., 60, 4915-4924 (1992).

42. Jobling, M.G., and Holmes, R.K., Mol. Microbiol., 5, 1755-1767 (1991).

43. Kunkel, T.A., Proc. Natl. Acad. Sci., USA, 82, 488-492 (1985).

44. Sanger, F., et al., Proc. Natl. Acad. Sci., USA, 74, 5463-5467 (1977).

45. Tartof, K.D., and Hobbs, C.A., Focus, 9, 12 (1987).

46. Karasic, R., et al., Ped. Inf. Dis. J., 8 (Suppl.), S62-65 (1988).

47. Hansen, E.J., et al., Infect. Immun., 56, 182-190 (1988).

48. Laemmli, U.K., Nature (London), 227, 680-685 (1970).

49. Ishida, S.I., et al., J. Clin. Microbiol., 34, 1694-1700 (1996)..

50. Burns, J.W., et al., Science, 272, 104-107 (1996).

51. McNeal, M.M., and Ward, R.L., Virology, 211, 474-480 (1995).

What is claimed is:

1. An antigenic composition comprising a selected antigen from a pathogenic bacterium, virus, fungus or parasite and an effective adjuvanting amount of a mutant cholera holotoxin, wherein the holotoxin has reduced toxicity compared to a wild-type cholera holotoxin and has a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the cholera holotoxin, wherein said holotoxin enhances the immune response in a vertebrate host to said antigen.

2. The antigenic composition of Claim 1 comprising more than one antigen.

3. The antigenic composition of Claim 1 wherein the amino acid at position 29 is histidine.

4. The antigenic composition of Claim 1 where the selected antigen is the *Haemophilus influenzae* P4 outer membrane protein, the *Haemophilus influenzae* P6 outer membrane protein, the *Haemophilus influenzae* adherence and penetration protein (Hap_S), the *Helicobacter pylori* urease protein, the *Neisseria meningitidis* Group B recombinant class 1 pilin (rpilin), the *Neisseria meningitidis* Group B class 1 outer membrane protein (PorA), the respiratory syncytial virus fusion protein, a rotavirus virus-like particle or the herpes simplex virus (HSV) type 2 glycoprotein D (gD2).

5. The antigenic composition of Claim 4 where the selected antigen is the *Haemophilus influenzae* P4 outer membrane protein, the *Haemophilus influenzae* P6 outer membrane protein, the *Haemophilus influenzae* Hap_S protein or any combination thereof.

6. The antigenic composition of Claim 4 where the selected antigen is the *Helicobacter pylori* urease protein.

7. The antigenic composition of Claim 4 where the selected antigen is the *Neisseria meningitidis* rpilin, the *Neisseria meningitidis* PorA protein or a combination thereof.

8. The antigenic composition of Claim 4 where the selected antigen is the respiratory syncytial virus fusion protein.

9. The antigenic composition of Claim 4 where the selected antigen is a rotavirus virus-like particle.

10. The antigenic composition of Claim 9 where the virus-like particle is a rotavirus 2/6-virus-like particle.

11. The antigenic composition of Claim 4 where the selected antigen is HSV gD2.

12. The antigenic composition of Claim 11 where the antigenic composition is a polynucleotide vaccine comprising plasmid DNA encoding HSV gD2.

13. The antigenic composition of Claim 1 which further comprises a diluent or carrier.

14. The antigenic composition of Claim 1 which further comprises a second adjuvant in addition to the mutant cholera holotoxin.

15. The antigenic composition of Claim 1 wherein at least one additional mutation is made to the A subunit of the cholera holotoxin at a position other than amino acid 29.

16. The antigenic composition of Claim 15 wherein the at least one additional mutation is made as a substitution for the arginine at amino acid 7, the aspartic acid at position 9, the arginine at position 11, the histidine at position 44, the valine at position 53, the arginine at position 54, the serine at position 61, the serine at position 63, the histidine at position 70, the valine at position 97, the tyrosine at position 104, the proline at position 106, the histidine at position 107, the serine at position 109, the glutamic acid at position 110, the glutamic acid at position 112, the serine at position 114, the tryptophan at position 127, the arginine at position 146 and the arginine at position 192.

17. A method for increasing the ability of an antigenic composition containing a selected antigen from a pathogenic bacterium, virus, fungus or parasite to elicit the immune response of a vertebrate host, which comprises administering to said host an antigenic composition of Claim 1.

18. A method for increasing the ability of an antigenic composition containing an *Haemophilus influenzae* antigen to elicit the immune response of a vertebrate host, which comprises administering to said host an antigenic composition of Claim 5.

19. A method for increasing the ability of an antigenic composition containing a *Helicobacter pylori* antigen to elicit the immune response of a vertebrate host, which comprises administering to said host an antigenic composition of Claim 6.

20. A method for increasing the ability of an antigenic composition containing a *Neisseria meningitidis* antigen to elicit the immune response of a vertebrate host, which comprises administering to said host an antigenic composition of Claim 7.

21. A method for increasing the ability of an antigenic composition containing a respiratory syncytial virus antigen to elicit the immune response of a vertebrate host, which comprises administering to said host an antigenic composition of Claim 8.

22. A method for increasing the ability of an antigenic composition containing a rotavirus antigen to elicit the immune response of a vertebrate host, which comprises administering to said host an antigenic composition of Claim 9.

23. A method for increasing the ability of an antigenic composition containing a herpes simplex virus antigen to elicit the immune response of a vertebrate host, which comprises administering to said host an antigenic composition of Claim 11.

24. A plasmid containing an isolated and purified DNA sequence comprising a DNA sequence which encode an immunogenic mutant cholera holotoxin having a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the cholera holotoxin, and wherein the DNA sequence is operatively linked to an arabinose inducible promoter.

25. A host cell transformed, transduced or transfected with the plasmid of Claim 24.

26. A method of producing an immunogenic mutant cholera holotoxin, wherein the cholera holotoxin has reduced toxicity compared to a wild-type cholera holotoxin and has a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the cholera holotoxin, which comprises transforming, transducing or transfecting a host cell with the plasmid of Claim 24 and culturing the host cell under conditions which permit the expression of said recombinant immunogenic detoxified protein by the host cell.

27. Use of effective adjuvanting amount of a mutant cholera holotoxin, wherein the holotoxin has reduced toxicity compared to a wild-type cholera holotoxin and has a substitution other than aspartic acid for the glutamic acid at position 29 of the A subunit of the cholera holotoxin, in combination with a selected antigen from a pathogenic bacterium, virus, fungus or parasite, to prepare an antigenic composition, wherein said holotoxin enhances the immune response in a vertebrate host to said antigen.

PCT

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(71) Applicants *(for all designated States except US)*: AMERICAN CYANAMID COMPANY [US/US]; Five Giralda Farms, Madison, NJ 07940 (US). THE GOVERNMENT OF THE UNITED STATES OF AMERICA as represented by the UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES [US/US]; 4301 Jones Bridge Road, Bethesda, MD 20814–4799 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: HOLMES, Randall, K. [US/US]; 23371 Morning Rose Drive, Golden, CO 80401 (US). JOBLING, Michael, G. [GB/US]; 5250 Cherry Creek Drive South, Apt. 18A, Denver, CO 80222 (US). ELDRIDGE, John, H. [US/US]; 4 Beatrice Cove, Fairport, NY 14450 (US). GREEN, Bruce, A. [US/US]; 49 Northfield Gate, Pittsford, NY 14534 (US). HANCOCK, Gerald, E. [US/US]; 50 Plains Road, Honeoye Falls, NY 14472 (US). PEEK, Joel, A. [US/US]; 156 Railroad Mills Road, Pittsford, NY 14534 (US).

(74) Agents: GORDON, Alan, M.; American Home Products Corporation, Patent Law Dept. – 2B2, One Campus Drive, Parsippany, NJ 07054 (US) et al.

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(54) Title: MUTANT CHOLERA HOLOTOXIN AS AN ADJUVANT

(57) Abstract

A mutant cholera holotoxin featuring a point mutation at amino acid 29 of the A subunit, wherein the glutamic acid residue is replaced by an amino acid other than aspartic acid, is useful as an adjuvant in an antigenic composition to enhance the immune response in a vertebrate host to a selected antigen from a pathogenic bacterium, virus, fungus or parasite. In a particular embodiment, the amino acid 29 is histidine. The mutant cholera holotoxin may contain at least one additional mutation in the A subunit at a position other than amino acid 29. The antigenic composition may include a second adjuvant in addition to the mutant cholera holotoxin.

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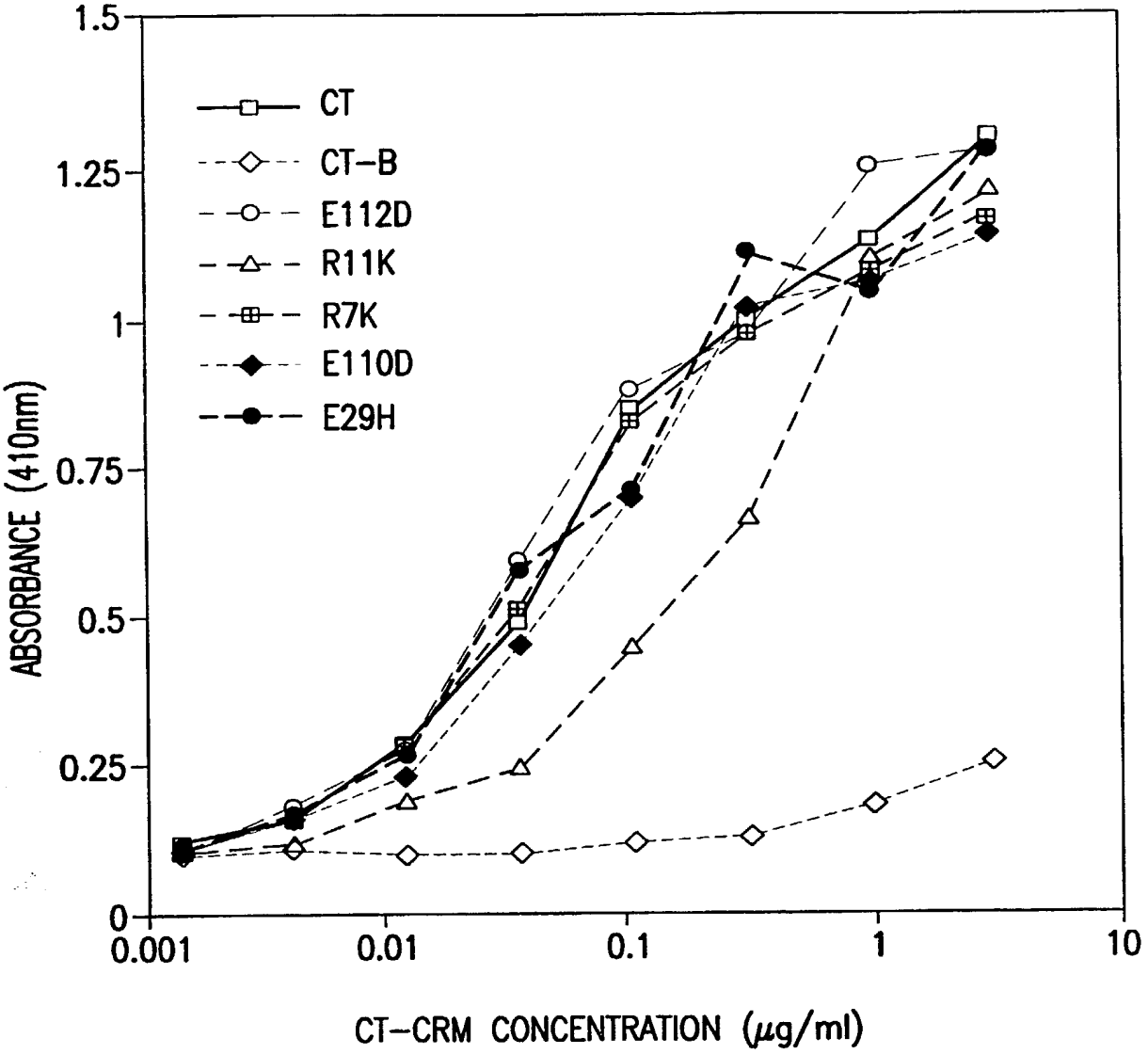

1.5
1.25
1
0.75
0.5
0.25
0
ABSORBANCE (410nm)
CT
CT-B
E112D
R11K
R7K
E110D
E29H
0.001
0.01
0.1
1
10
CT-CRM CONCENTRATION (μg/ml)

FIG. 1

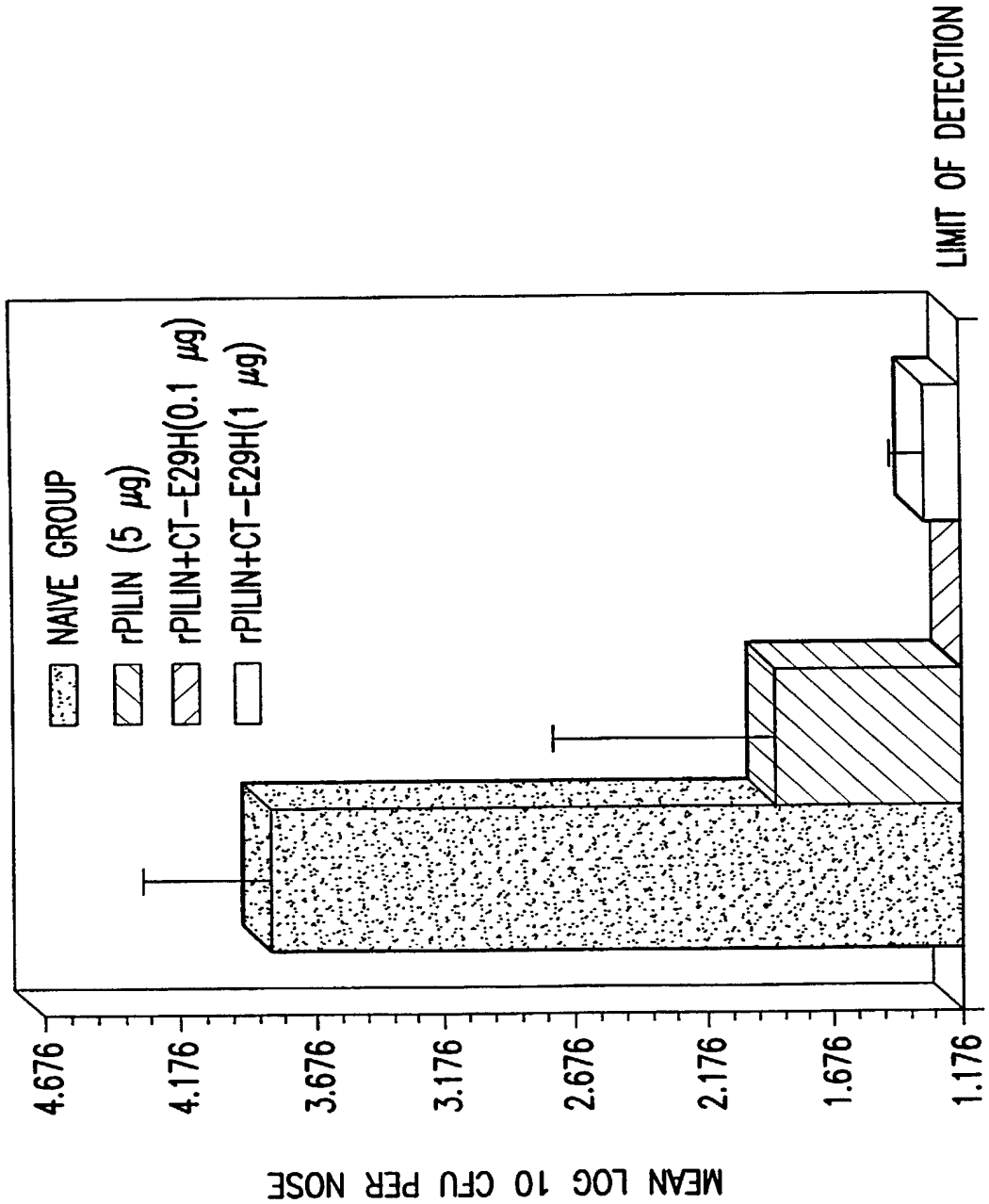

LIMIT OF DETECTION
NAIVE GROUP
rPILIN (5 μg)
rPILIN+CT-E29H(0.1 μg)
rPILIN+CT-E29H(1 μg)
4.676
4.176
3.676
3.176
2.676
2.176
1.676
1.176
MEAN LOG 10 CFU PER NOSE

FIG. 2

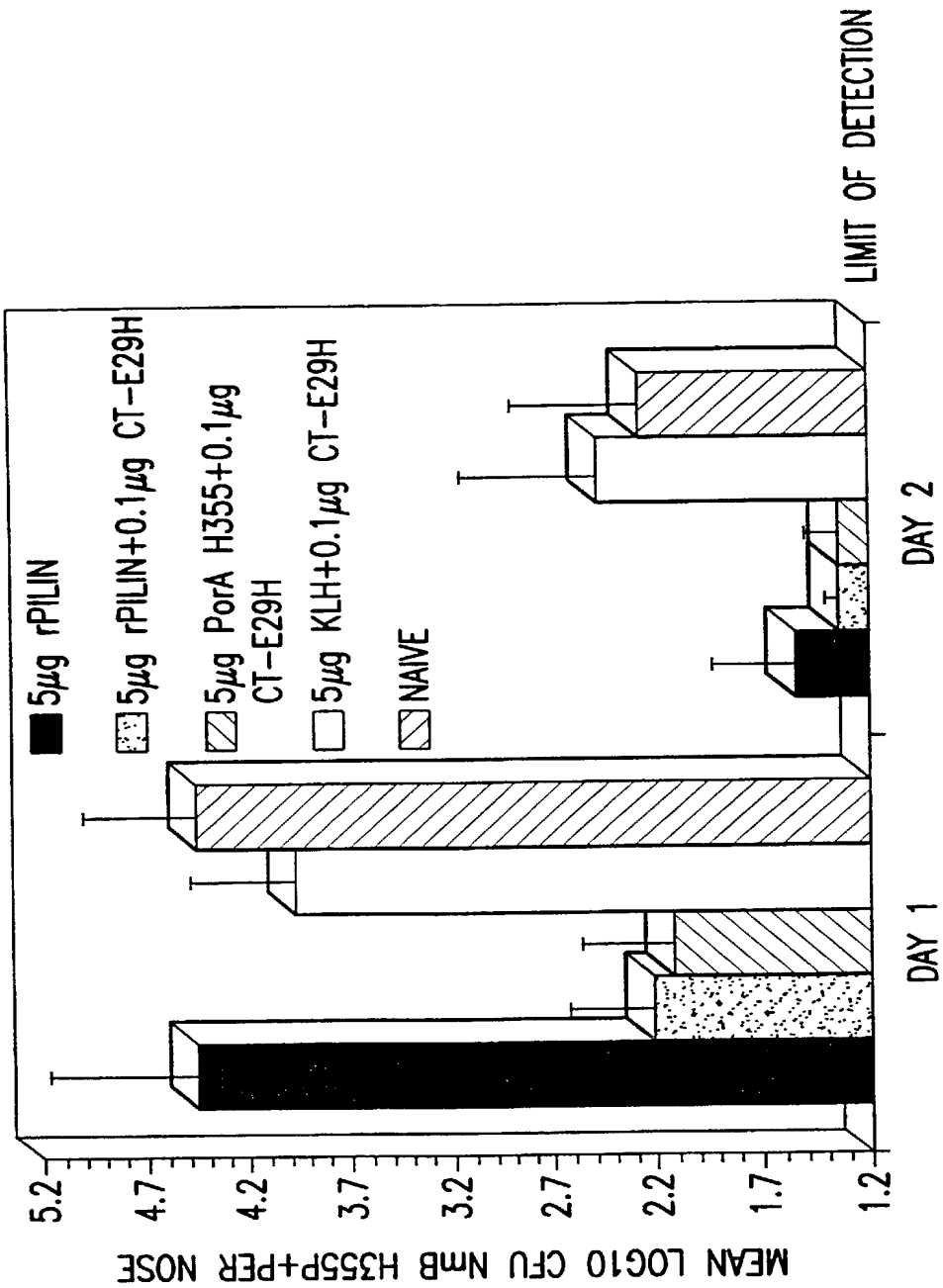

MEAN LOG10 CFU NmB H355P+PER NOSE
5.2
4.7
4.2
3.7
3.2
2.7
2.2
1.7
1.2
DAY 1
DAY 2
LIMIT OF DETECTION
5μg rPILIN
5μg rPILIN+0.1μg CT-E29H
5μg PorA H355+0.1μg CT-E29H
5μg KLH+0.1μg CT-E29H
NAIVE

FIG. 3

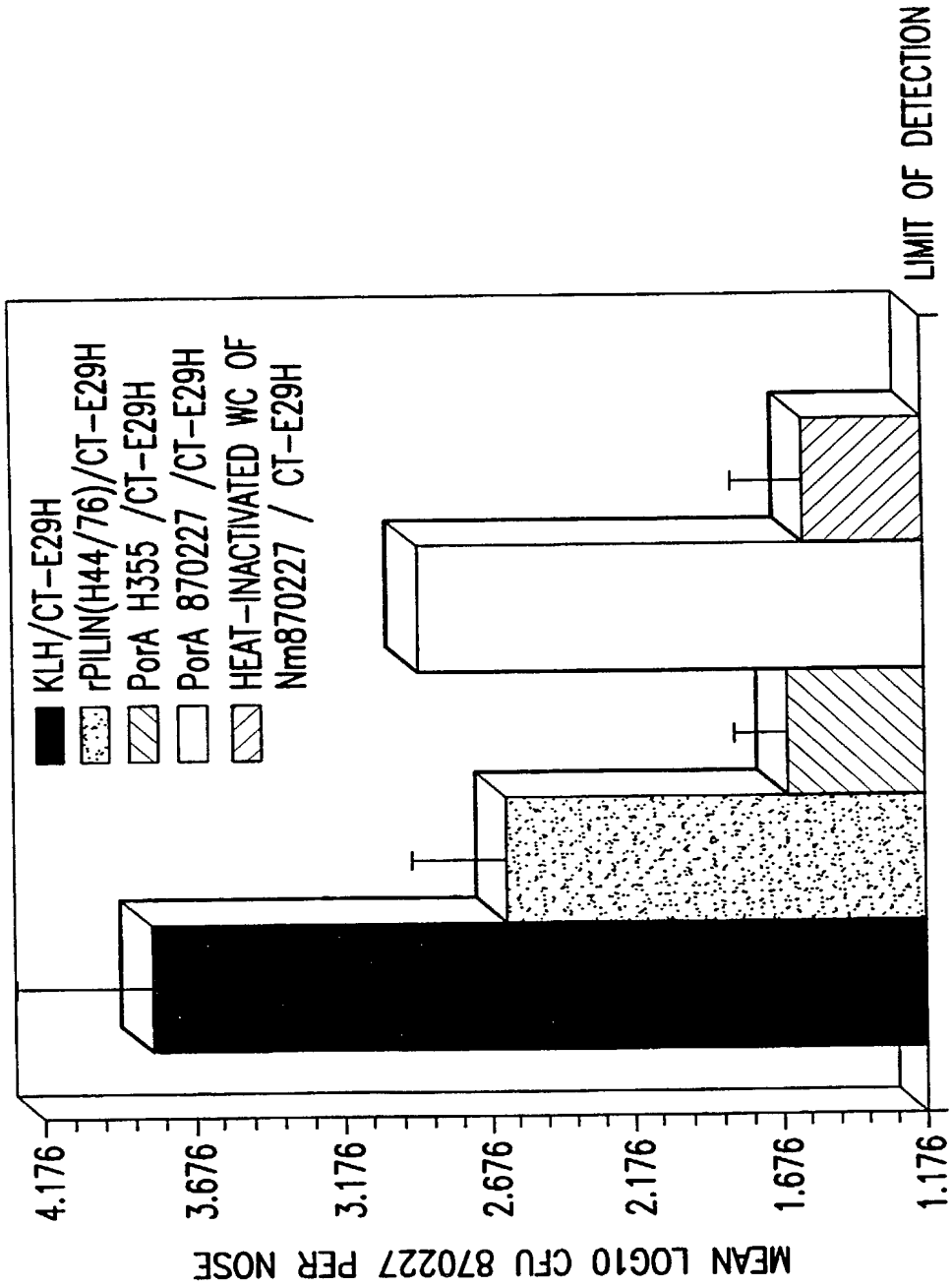

KLH/CT-E29H
rPILIN(H44/76)/CT-E29H
PorA H355 /CT-E29H
PorA 870227 /CT-E29H
HEAT-INACTIVATED WC OF Nm870227 / CT-E29H
LIMIT OF DETECTION
4.176
3.676
3.176
2.676
2.176
1.676
1.176
MEAN LOG10 CFU 870227 PER NOSE

FIG. 4

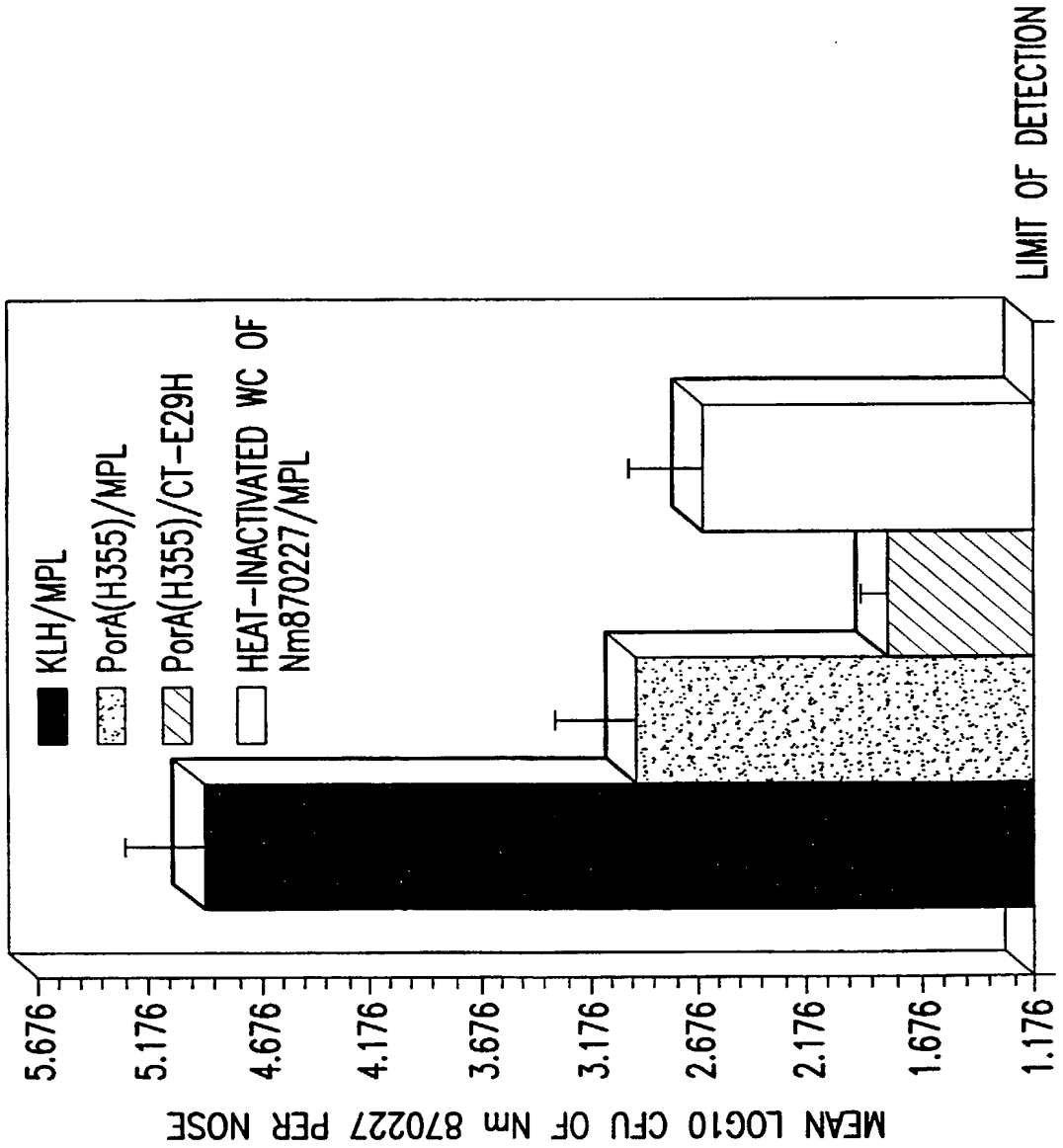

KLH/MPL
PorA(H355)/MPL
PorA(H355)/CT-E29H
HEAT-INACTIVATED WC OF Nm870227/MPL
LIMIT OF DETECTION
MEAN LOG10 CFU OF Nm 870227 PER NOSE
5.676
5.176
4.676
4.176
3.676
3.176
2.676
2.176
1.676
1.176

FIG.5

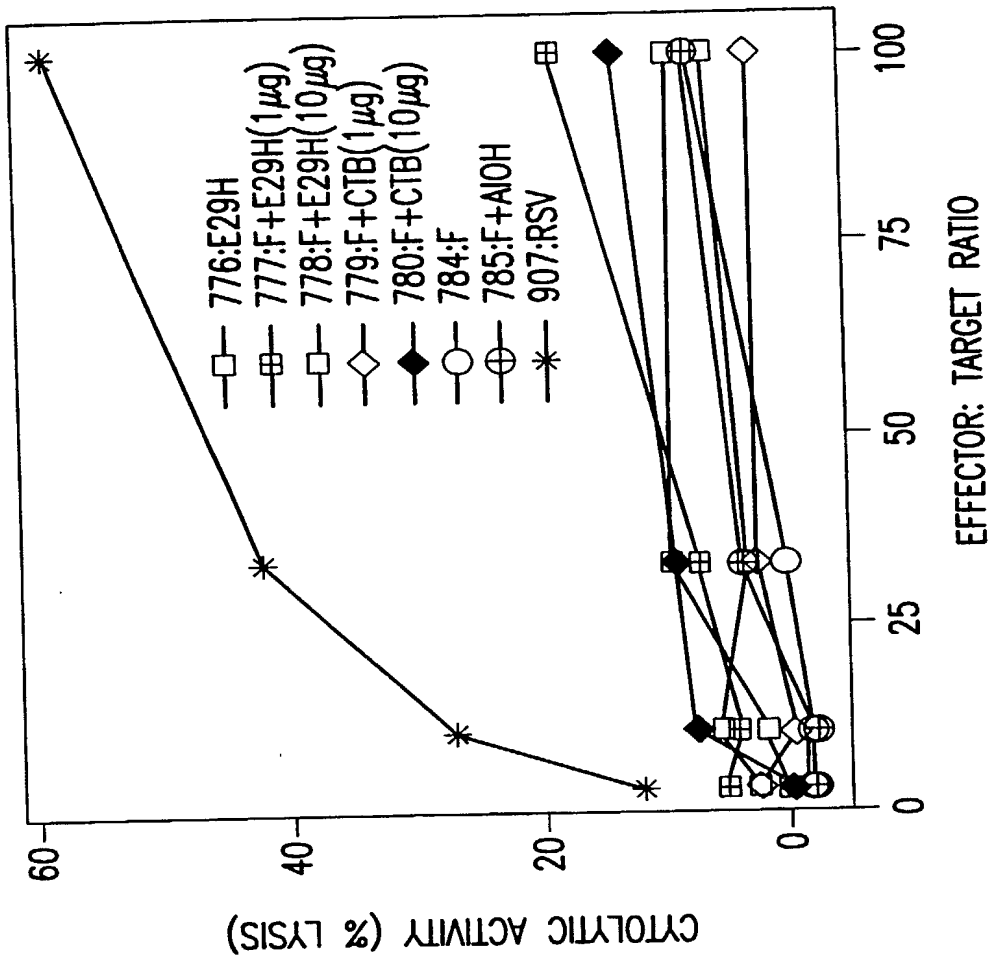

776:E29H
777:F+E29H(1μg)
778:F+E29H(10μg)
779:F+CTB(1μg)
780:F+CTB(10μg)
784:F
785:F+AlOH
907:RSV
CYTOLYTIC ACTIVITY (% LYSIS)
0
20
40
60
EFFECTOR: TARGET RATIO
0
25
50
75
100

FIG. 6

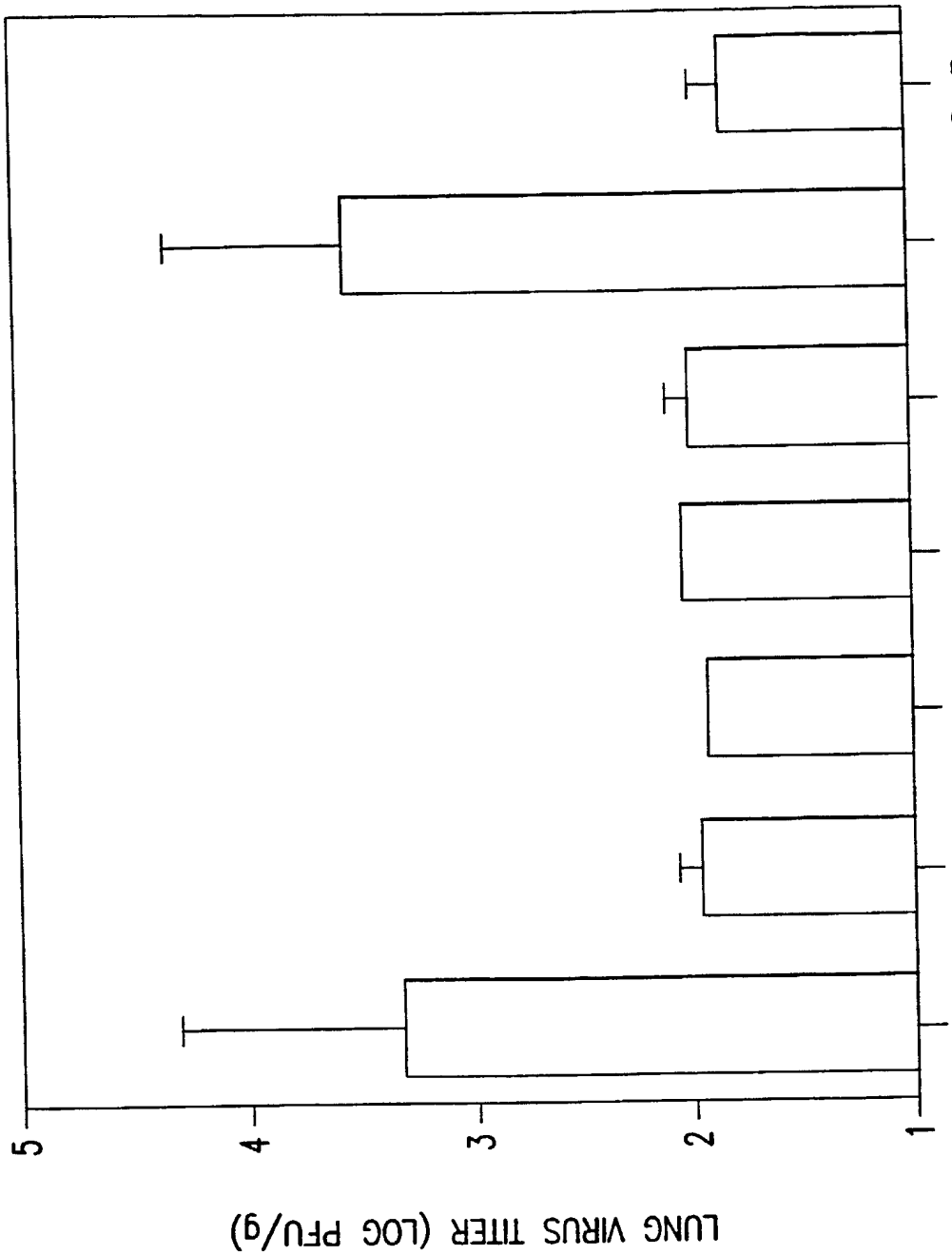

LUNG VIRUS TITER (LOG PFU/g)
5
4
3
2
1
PBS+ 10μg E29H
3μgF+ 1μg E29H
3μgF+ 10μg E29H
3μgF+ 1μg CTB
3μgF+ 10μg CTB
3μgF+ PBS
3μgF+ AlOH
VACCINE GROUP

FIG. 7

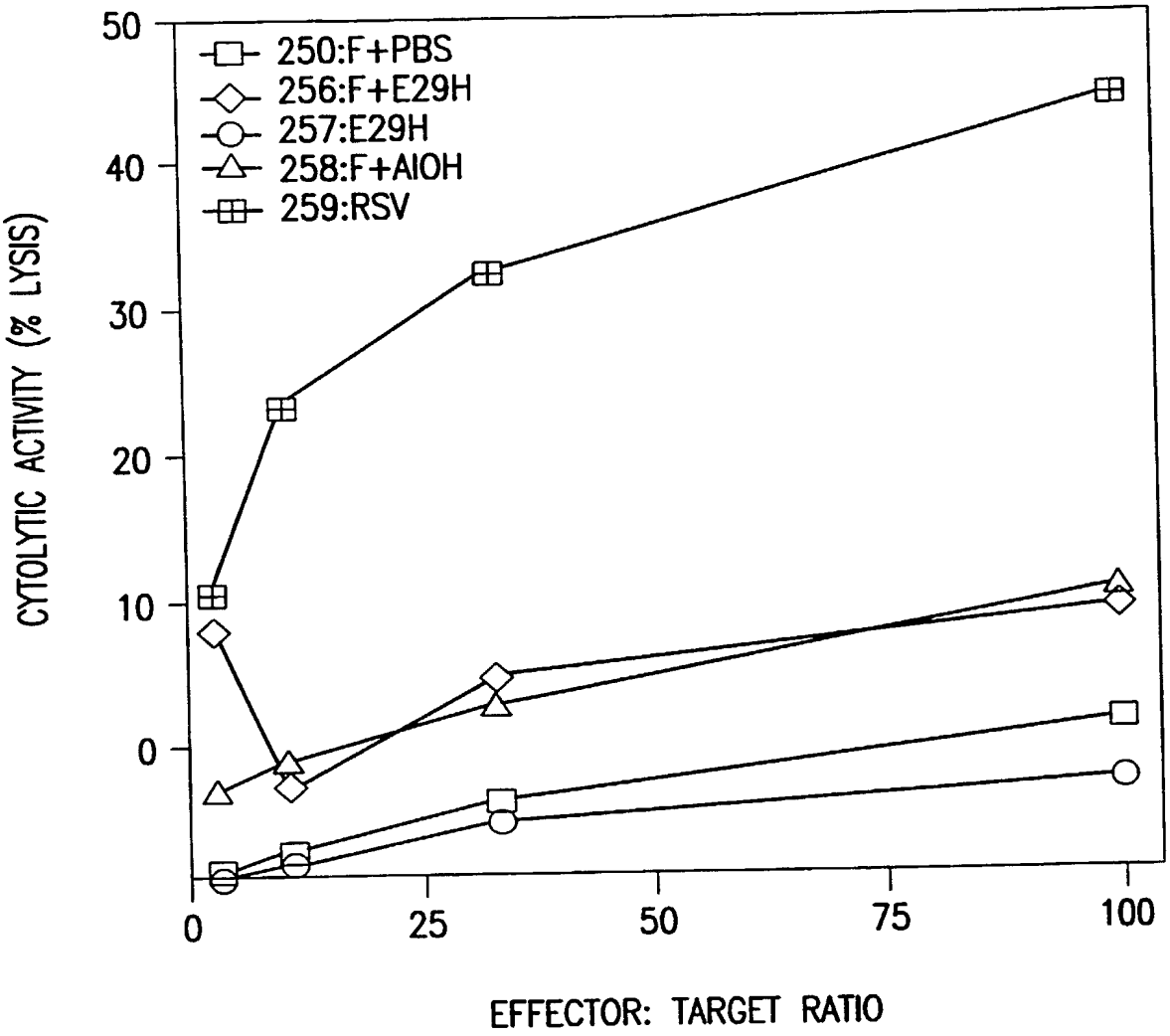

250:F+PBS
256:F+E29H
257:E29H
258:F+AIOH
259:RSV
CYTOLYTIC ACTIVITY (% LYSIS)
50
40
30
20
10
0
0
25
50
75
100
EFFECTOR: TARGET RATIO

FIG. 8

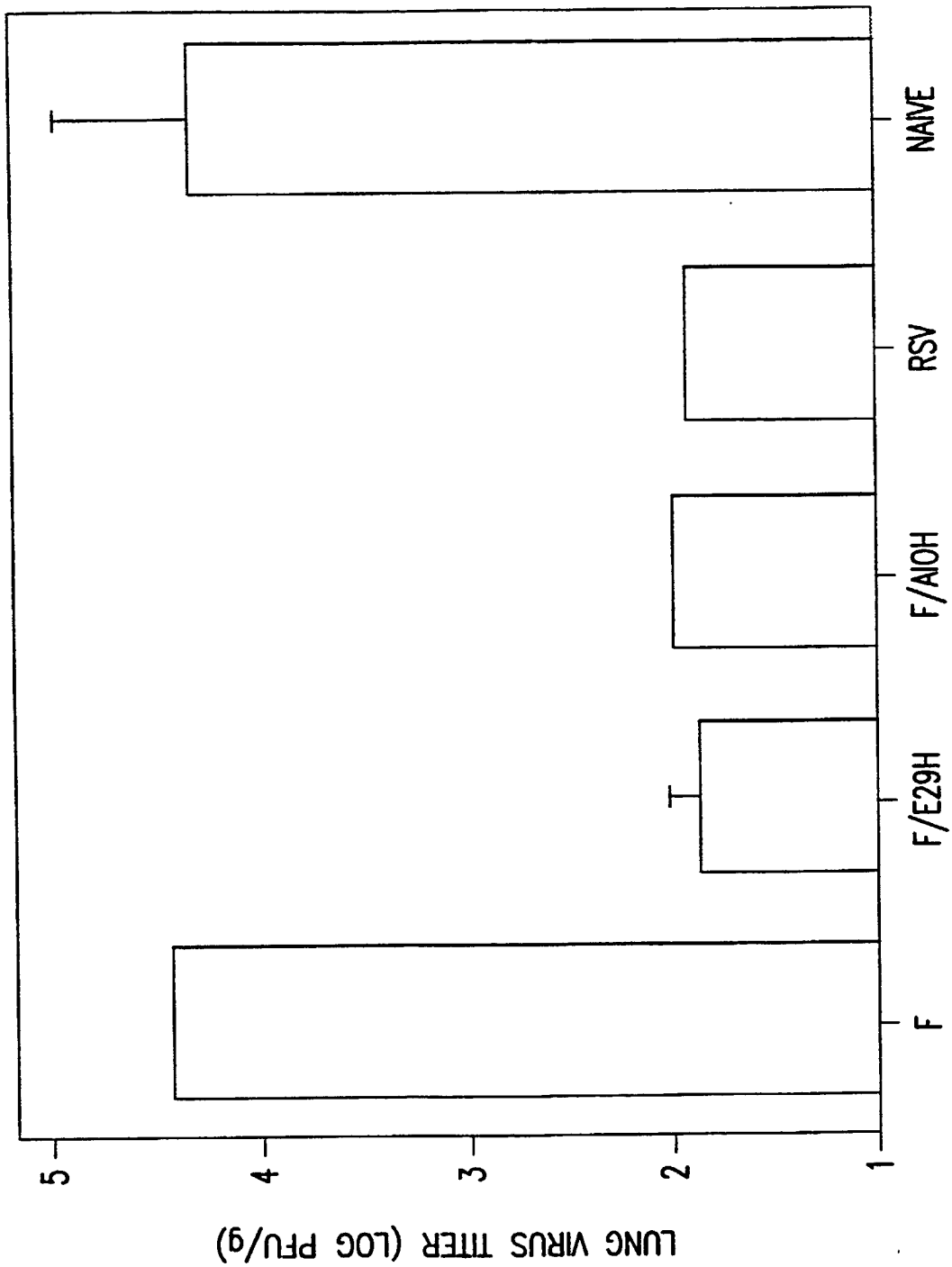
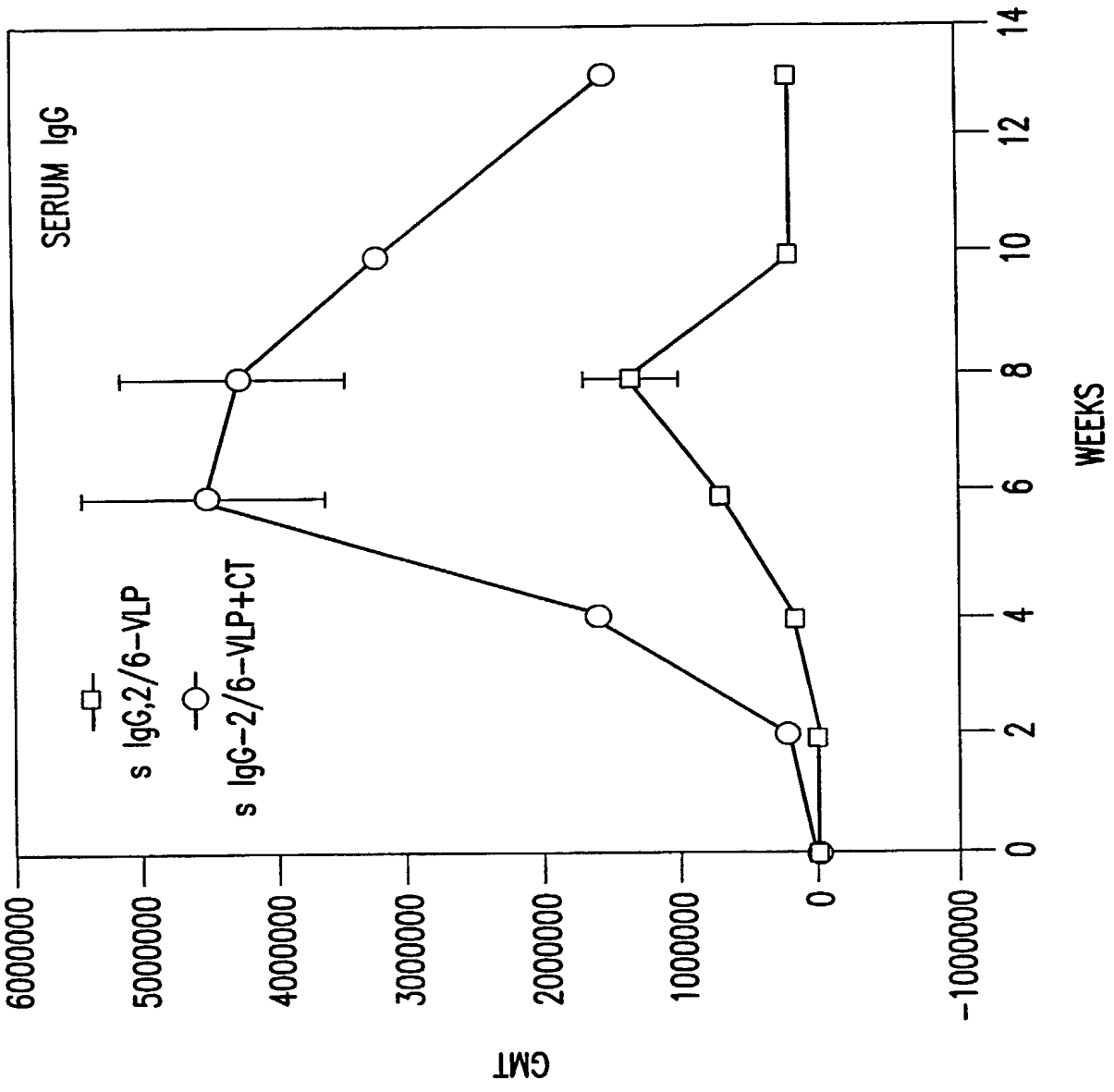

LUNG VIRUS TITER (LOG PFU/g)
5
4
3
2
1
F
F/E29H
F/AIOH
RSV
NAIVE
VACCINE GROUP

FIG. 9

FIG. 10A


SERUM IgG
s IgG,2/6-VLP
s IgG-2/6-VLP+CT
GMT
6000000
5000000
4000000
3000000
2000000
1000000
0
-1000000
0
2
4
6
8
10
12
14
WEEKS

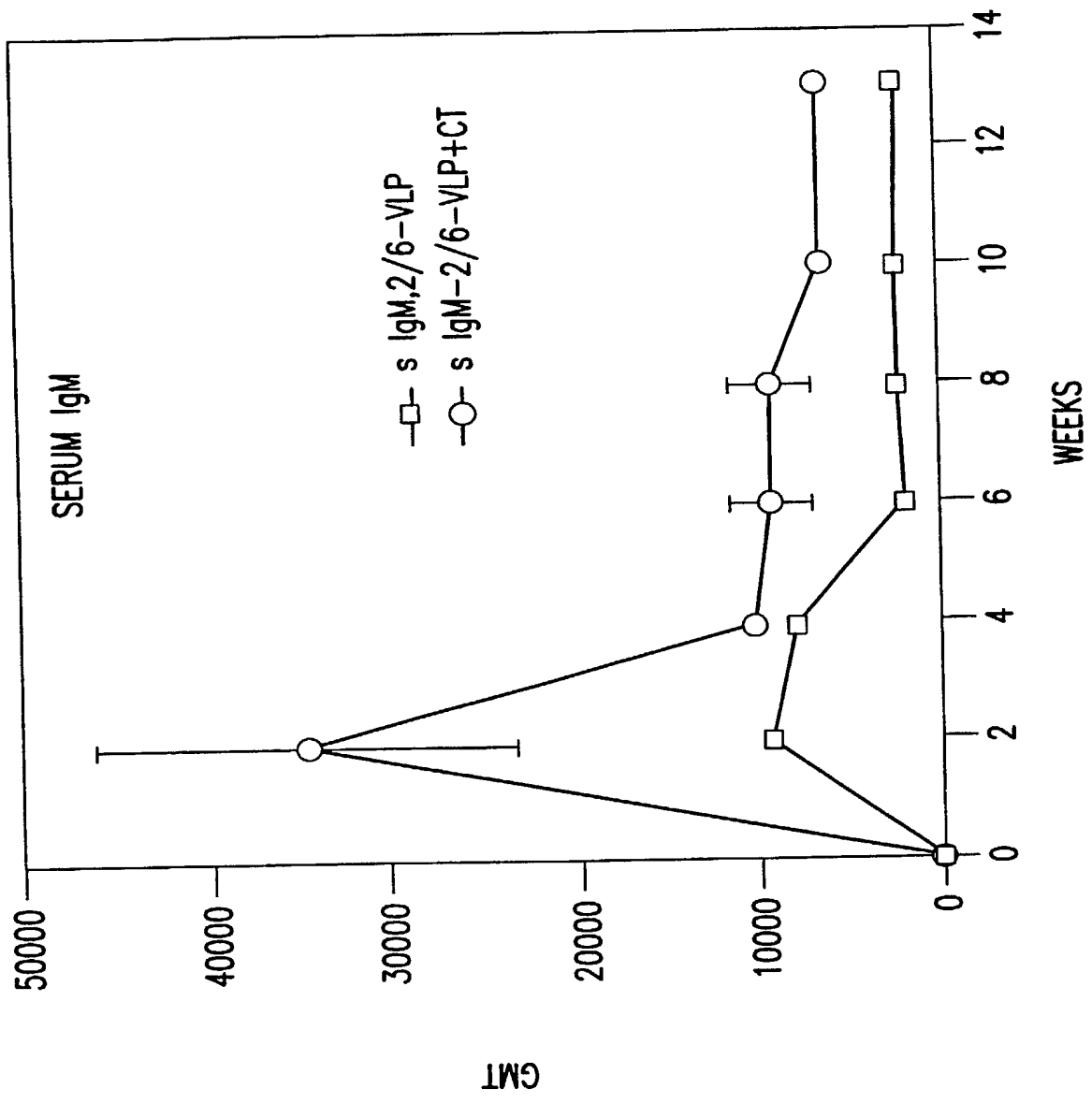


FIG. 10B

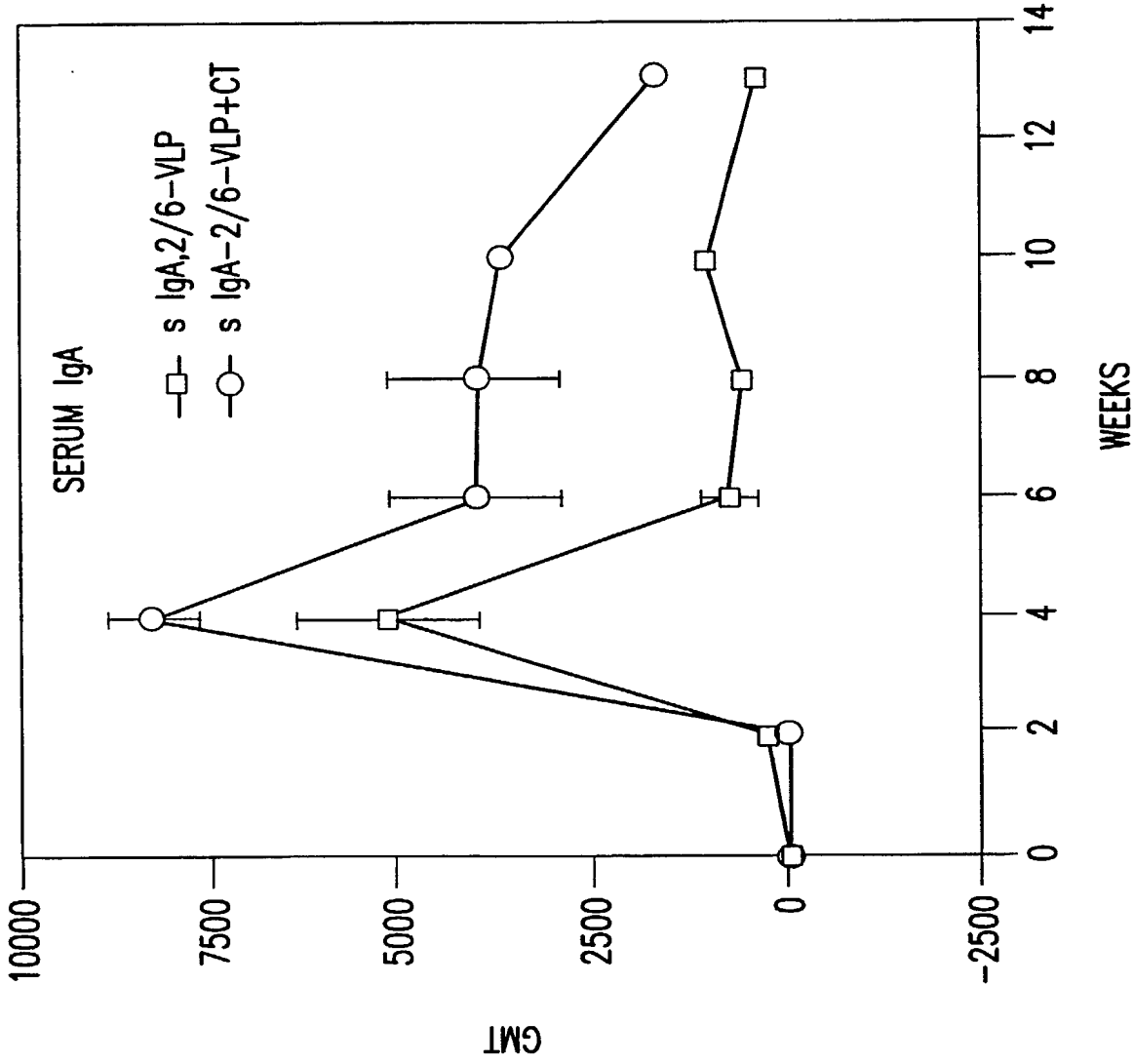

SERUM IgA
s IgA,2/6-VLP
s IgA-2/6-VLP+CT
GMT
10000
7500
5000
2500
0
-2500
0
2
4
6
8
10
12
14
WEEKS

FIG. 10C

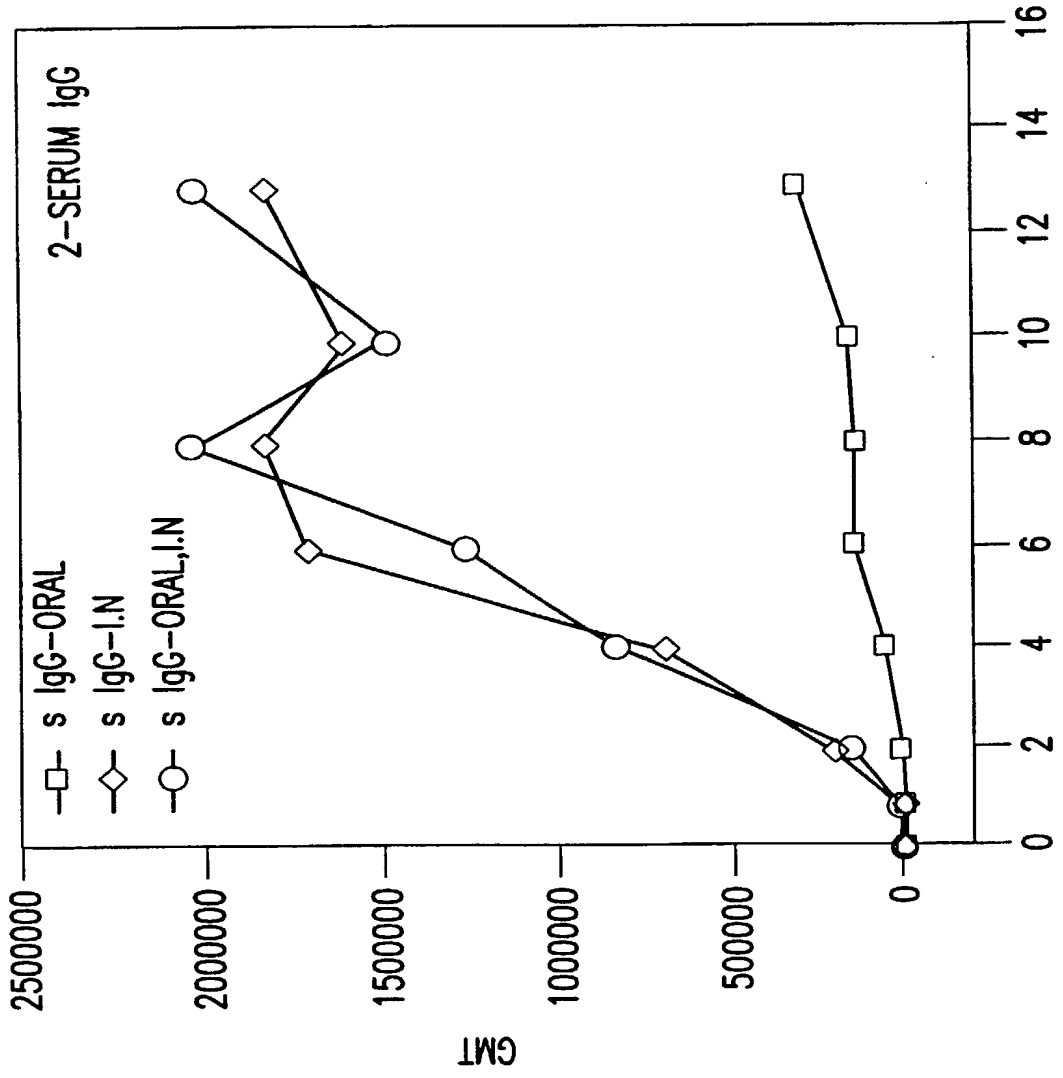

2-SERUM IgG
s IgG-ORAL
s IgG-I.N
s IgG-ORAL,I.N
GMT
2500000
2000000
1500000
1000000
500000
0
WEEKS
0
2
4
6
8
10
12
14
16

FIG. 11A

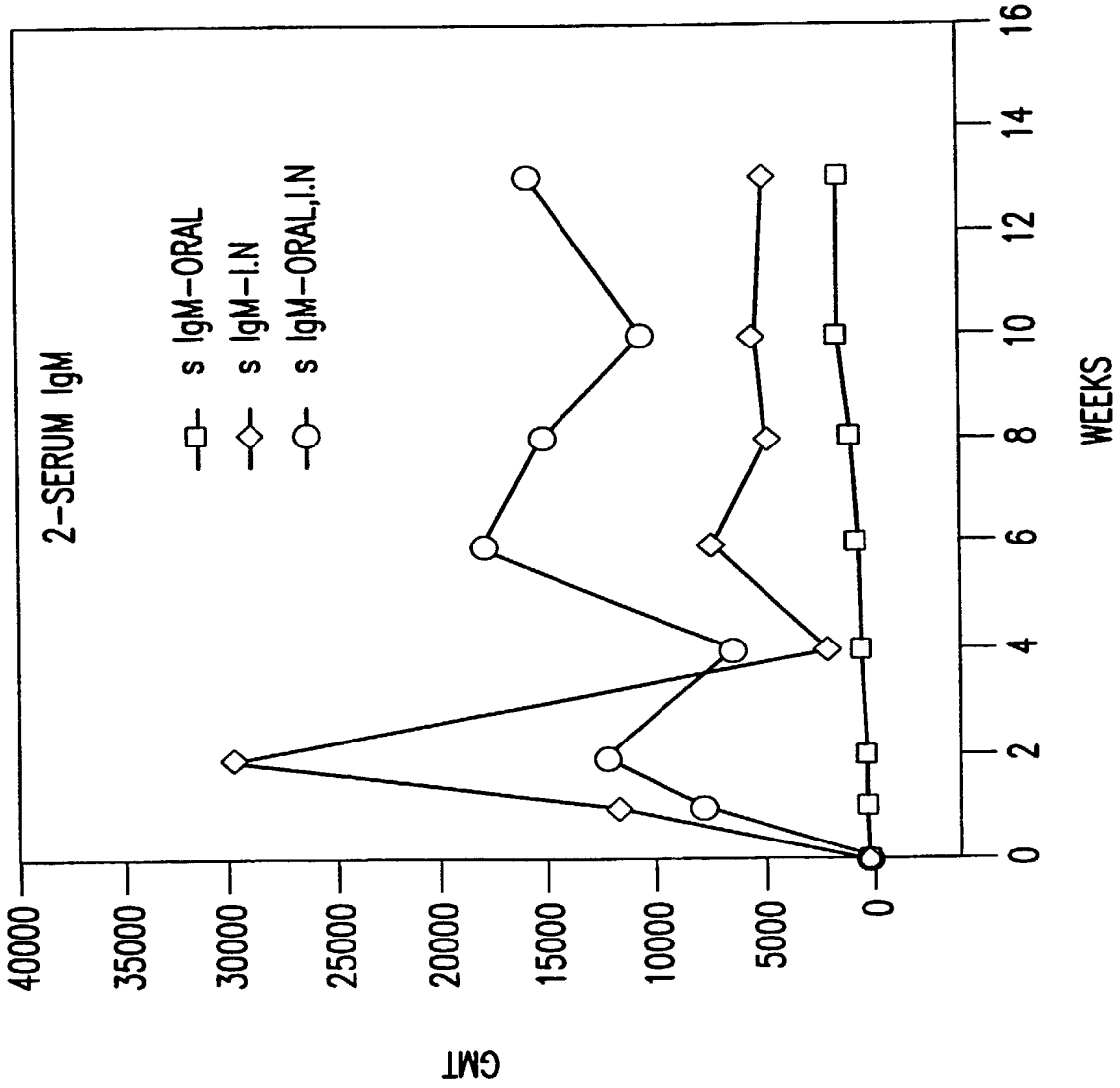

2-SERUM IgM
s IgM-ORAL
s IgM-I.N
s IgM-ORAL,I.N
GMT
40000
35000
30000
25000
20000
15000
10000
5000
0
0
2
4
6
8
10
12
14
16
WEEKS

FIG. 11B

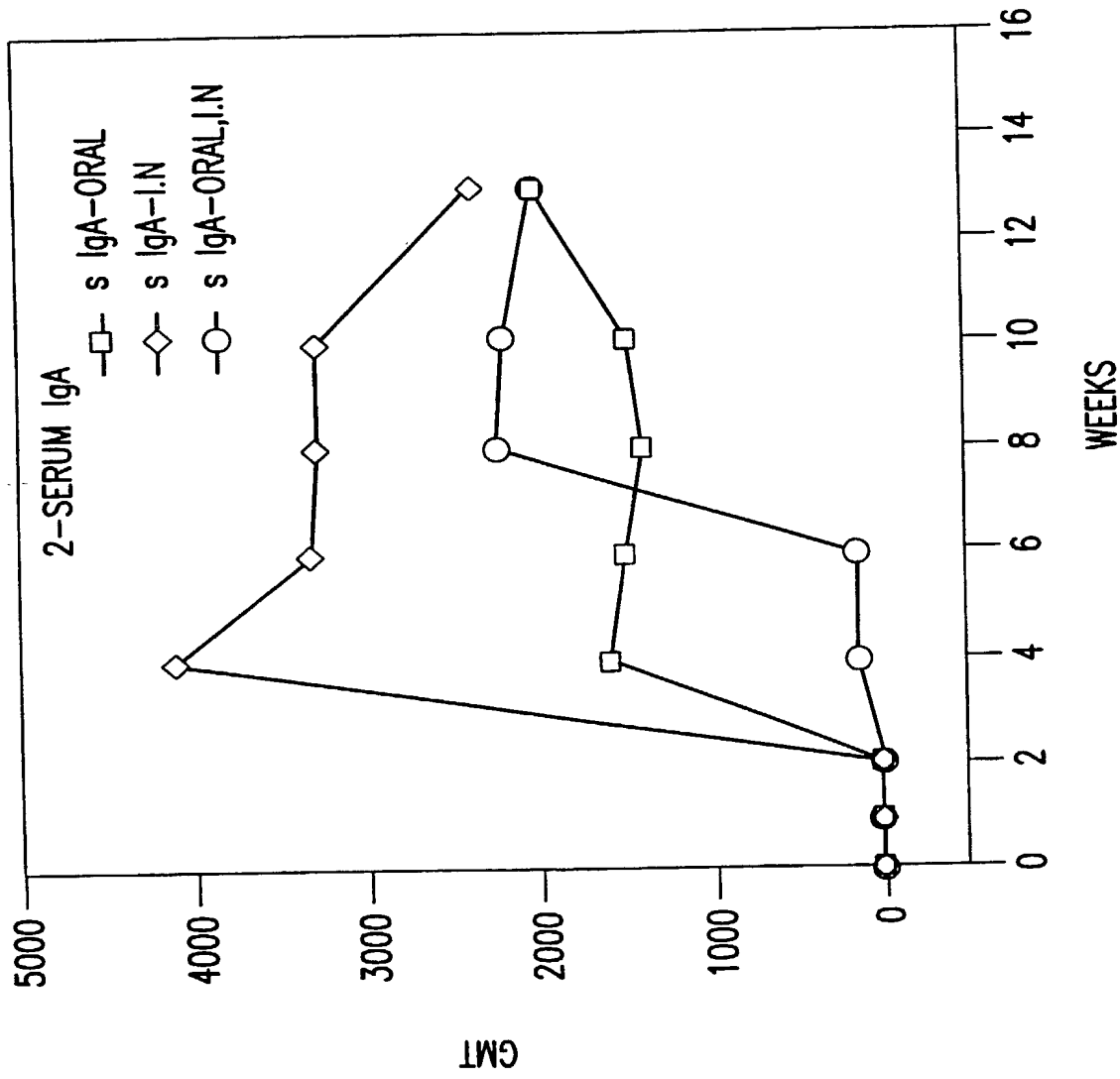

2-SERUM IgA
s IgA-ORAL
s IgA-I.N
s IgA-ORAL,I.N
GMT
5000
4000
3000
2000
1000
0
0
2
4
6
8
10
12
14
16
WEEKS

FIG. 11C

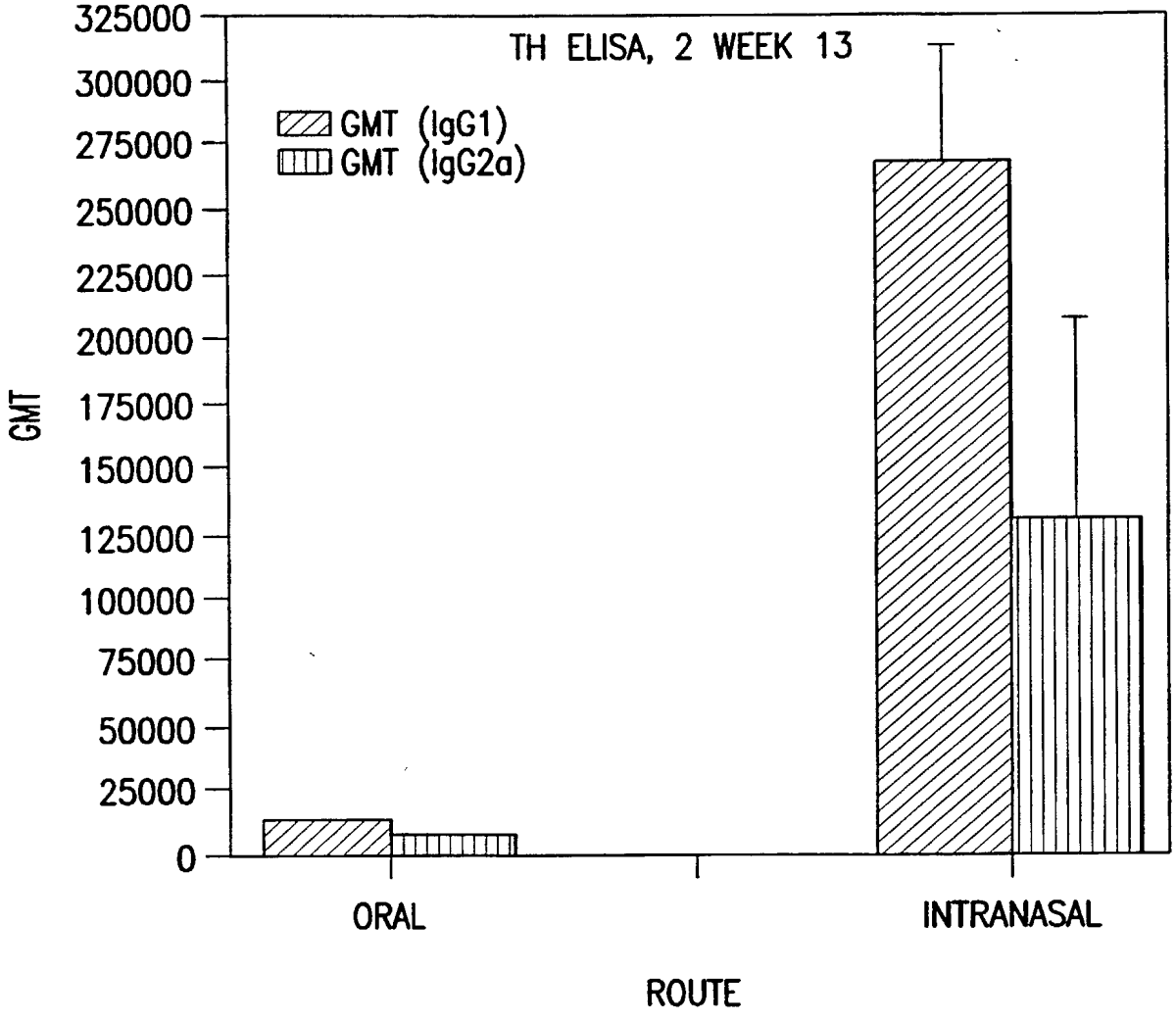

TH ELISA, 2 WEEK 13
GMT (IgG1)
GMT (IgG2a)
325000
300000
275000
250000
225000
200000
175000
150000
125000
100000
75000
50000
25000
0
GMT
ORAL
INTRANASAL
ROUTE

FIG. 12

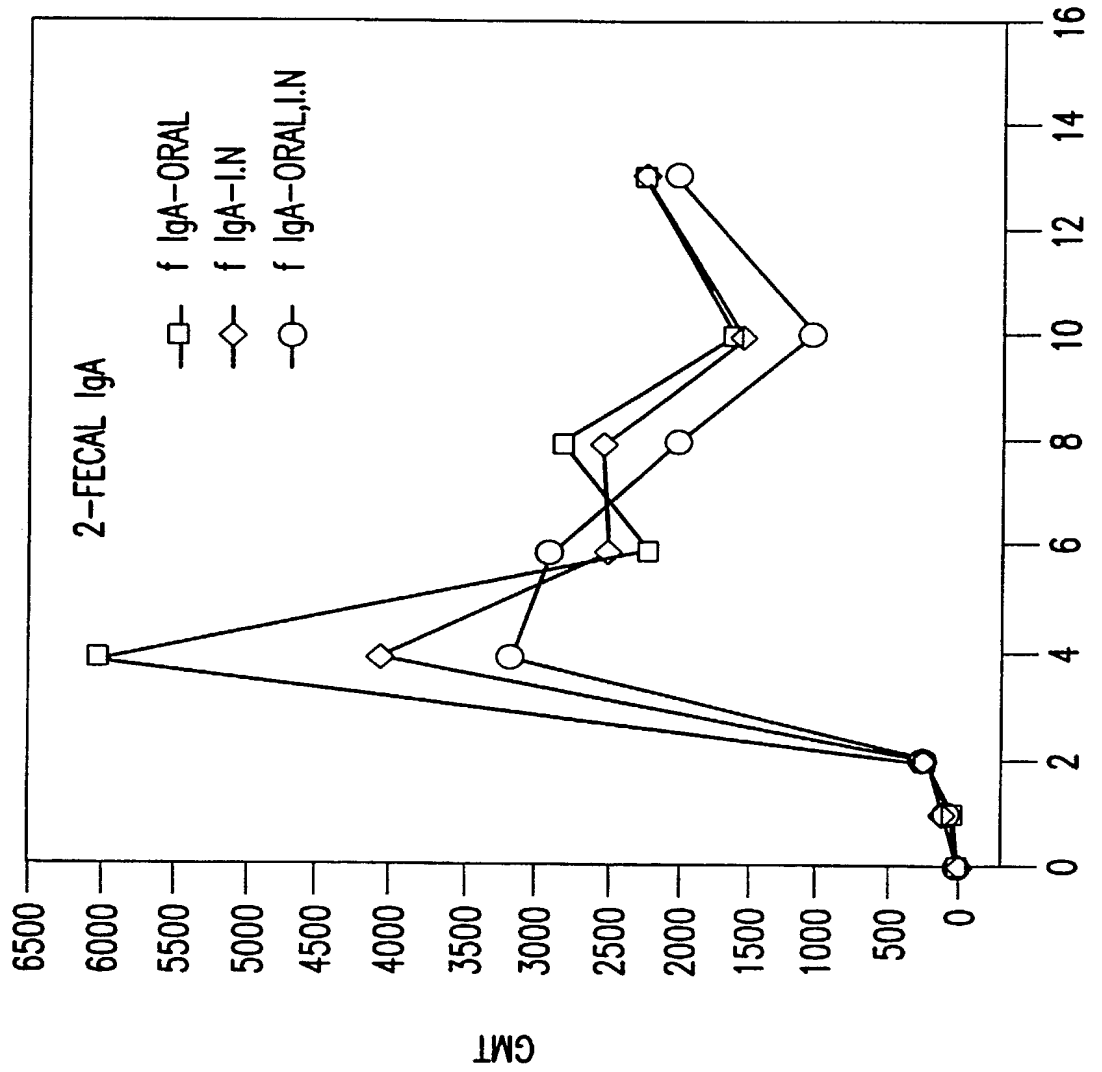

2-FECAL IgA
f IgA-ORAL
f IgA-I.N
f IgA-ORAL,I.N
GMT
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
16
WEEKS

FIG. 13A

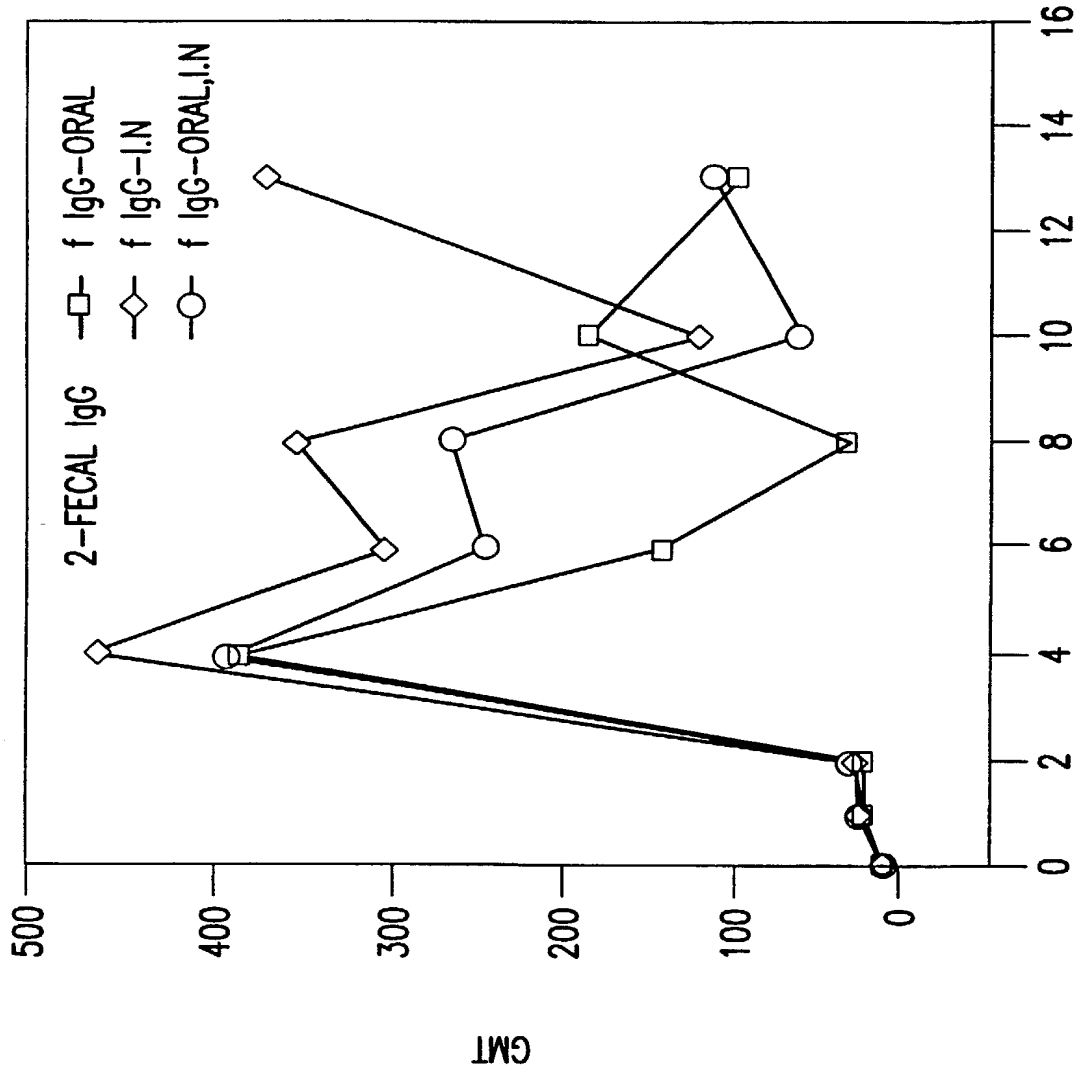

2-FECAL IgG
f IgG-ORAL
f IgG-I.N
f IgG-ORAL,I.N
GMT
500
400
300
200
100
0
0
2
4
6
8
10
12
14
16
WEEKS

FIG. 13B

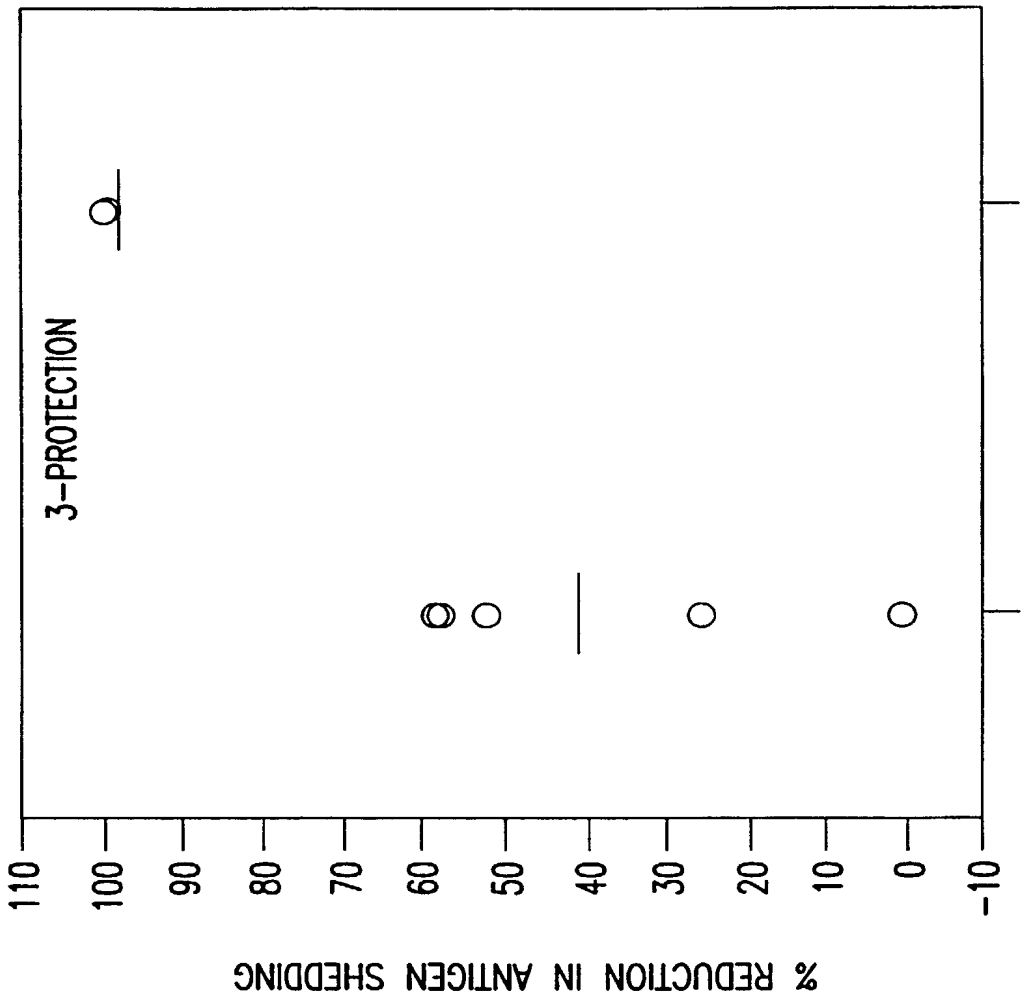

% REDUCTION IN ANTIGEN SHEDDING
110
100
90
80
70
60
50
40
30
20
10
0
-10
3-PROTECTION
2/6-VLP
n=5
2/6-VLP+CT CRMS
n=4
IMMUNIZATION

FIG. 14A

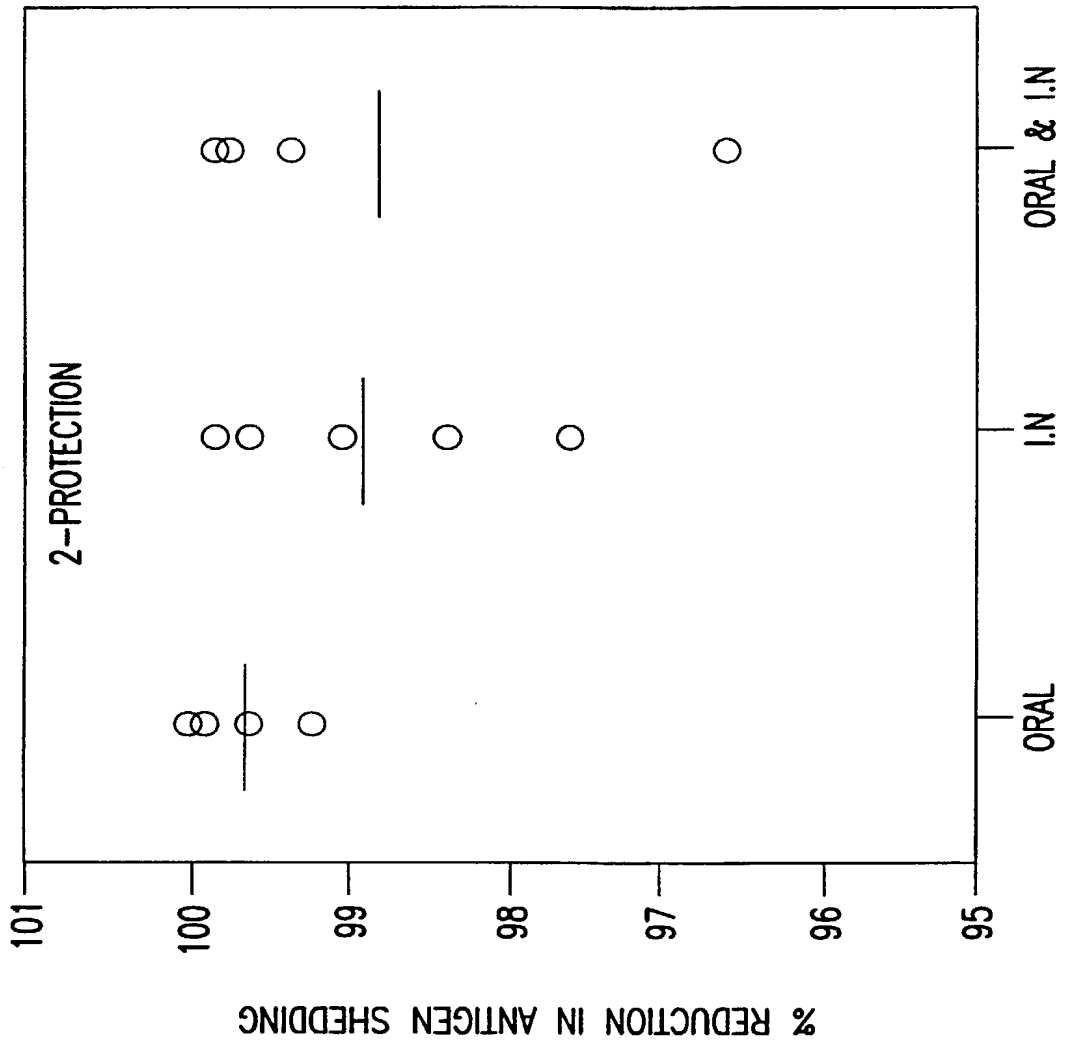

2-PROTECTION
% REDUCTION IN ANTIGEN SHEDDING
101
100
99
98
97
96
95
ORAL
I.N
ORAL & I.N
ROUTE

FIG. 14B

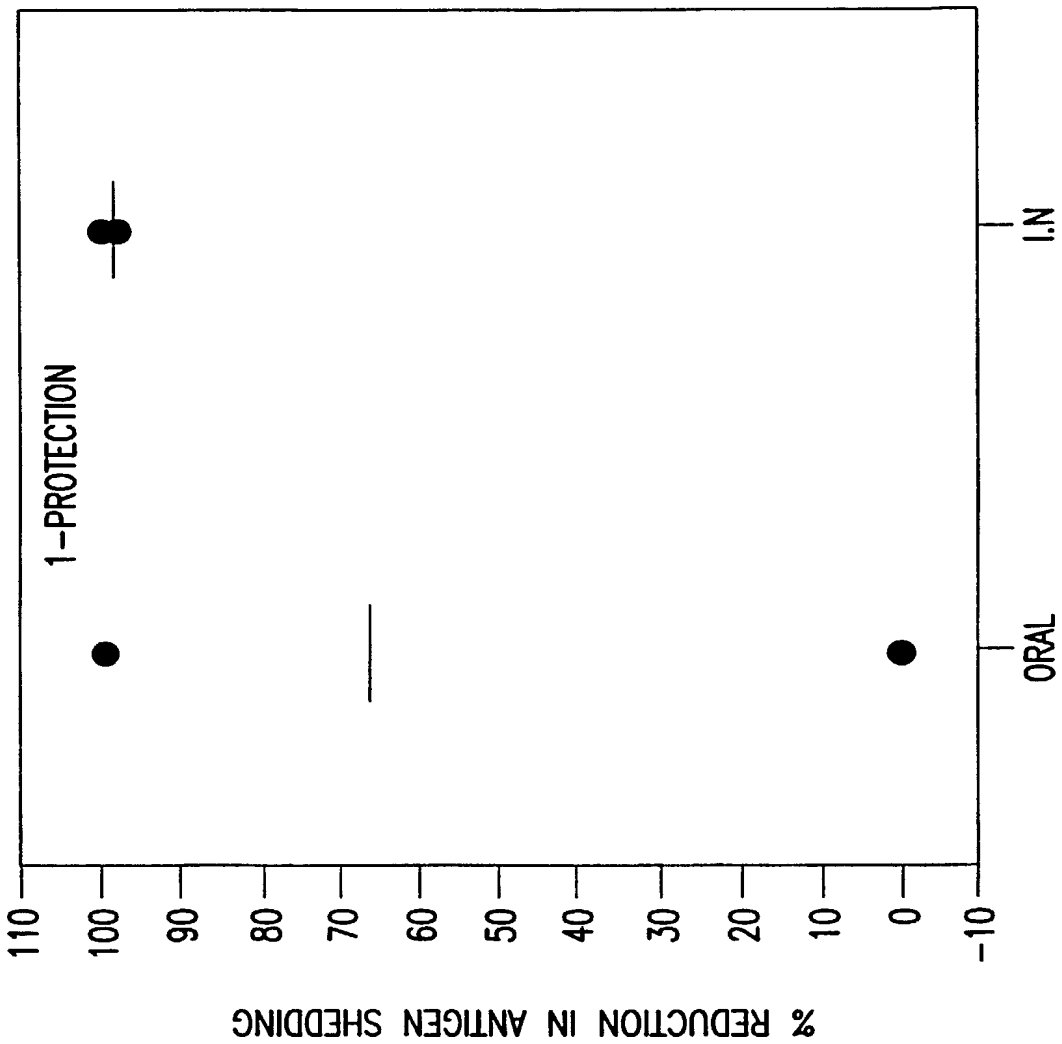

% REDUCTION IN ANTIGEN SHEDDING
110
100
90
80
70
60
50
40
30
20
10
0
-10
1-PROTECTION
ORAL
I.N
ROUTE

FIG. 14C

Docket No: 33,383-00
Patent

COMBINED DECLARATION AND POWER OF ATTORNEY
(Original, Design, Supplemental, Divisional, Continuation,CIP)

As the below named inventor, I hereby declare that:

INVENTORSHIP IDENTIFICATION

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

Mutant Cholera Holotoxin as an Adjuvant

SPECIFICATION IDENTIFICATION

the specification of which: (complete (a), (b), or (c))

(a) ☐ is attached hereto.
(b) ☐ was filed on as
☐ Serial Number
☐ Express Mail No. , as Serial Number not yet known
(c) ☒ was described and claimed in PCT International Application No. PCT/US99/22520 filed on September 30, 1999 and as amended under PCT Article 19 on (if any), which entered the U.S. national phase as Serial Number 09/806,370.

ACKNOWLEDGEMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37 CFR 1.56(a).

Docket No: 33,383-00
Patent

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventors certificate or of any PCT International application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate of any PCT International application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed.

(d) ☒ No such applications have been filed.
(e) ☐ Such applications have been filed as follows.

NOTE: Where item (c) is entered above and the International Application which designated the U.S. claimed priority, check item (e), enter the details below and make the priority claim.

Earliest Foreign Application(s), if any, filed within 12 months (6 months for Design) prior to this U.S. Application

Country	Application No.	Date of Filing (Day, Month, Year)	Priority Claimed 35 USC 119
PCT	PCT/US99/22520	30 September 1999	

All Foreign Application(s), if any, Filed More Than 12 Months
(6 Months for Design) Prior to This U.S. Application)

Docket No: 33,383-00
Patent

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. § 119(E))

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

PROVISIONAL APPLICATION NUMBER	**FILING DATE**
60/102,430	September 30, 1998

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)
(UNDER 35 U.S.C. 120)

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT International filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 USC 120

U.S. Applications		**Status (Check One)**		
U.S. Applications	U.S. Filing Date	Patented	Pending	Abandoned
		☐	☐	☐
		☐	☐	☐
		☐	☐	☐
		☐	☐	☐

Docket No: 33,383-00
Patent

PCT Applications Designating U.S.		
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NO. ASSIGNED (if any)
PCT/US99/22520	September 30, 1999	09/806,370

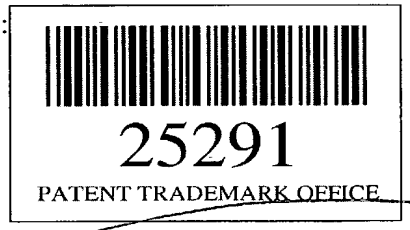
POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

☒ Customer Number: 25291 Bar Code:

☐ Attached as part of this declaration and power of attorney is the authorization of the above-named attorney(s) to accept and follow instructions from my representative(s).

SEND CORRESPONDENCE TO:
Customer Number: 25291 Bar Code:



DIRECT ALL TELEPHONE CALLS TO:
Name: Alan M. Gordon
Tel. No. (845) 602-4636

Docket No: 33,383-00
Patent

DECLARATION

I hereby declare that all statements herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE(S)

Full name of SOLE OR FIRST INVENTOR: **Randall K. Holmes**

Inventor's Signature Randall K. Holmes Date Sept. 10, 2001

Country of Citizenship: **United States of America**
Residence : **23371 Morning Rose Drive, Golden, Colorado 80401** CO
Post Office Address: **23371 Morning Rose Drive, Golden, Colorado 80401**

Full name of SECOND JOINT INVENTOR: **Michael G. Jobling**

Inventor's Signature M. G. Jobling. Date 9/10/01

Country of Citizenship: **United Kingdom**
Residence : **12743 East Wyoming Place, Aurora, Colorado 80012** CO
Post Office Address: **12743 East Wyoming Place, Aurora, Colorado 80012**

Full name of THIRD JOINT INVENTOR: **John H. Eldridge**

Inventor's Signature _____ Date _____

Country of Citizenship: **United States of America**
Residence : **4 Beatrice Cove, Fairport, New York 14450** NY
Post Office Address: **4 Beatrice Cove, Fairport, New York 14450**

Full name of FOURTH JOINT INVENTOR: **Bruce A. Green**

Inventor's Signature _____ Date _____

Country of Citizenship: **United States of America**
Residence : **49 Northfield Gate, Pittsford, New York 14534** NY
Post Office Address: **49 Northfield Gate, Pittsford, New York 14534**

Full name of FIFTH JOINT INVENTOR: **Gerald E. Hancock**

Inventor's Signature _____ Date _____

Country of Citizenship: **United States of America**
Residence : **50 Plains Road, Honeoye Falls, New York 14472** NY
Post Office Address: **50 Plains Road, Honeoye Falls, New York 14472**

SIGNATURE(S) (continued)

Full name of SIXTH JOINT INVENTOR: **Joel A. Peek**

Inventor's Signature _____ Date _____

Country of Citizenship: **United States of America**
Residence : **115 Moores Court, Brentwood, TN 37027**
Post Office Address: **115 Moores Court, Brentwood, TN 37027**

COMBINED DECLARATION AND POWER OF ATTORNEY
(Original, Design, Supplemental, Divisional, Continuation,CIP)

As the below named inventor, I hereby declare that:

INVENTORSHIP IDENTIFICATION

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

Mutant Cholera Holotoxin as an Adjuvant

SPECIFICATION IDENTIFICATION

the specification of which: (complete (a), (b), or (c))

(a) ☐ is attached hereto.

(b) ☐ was filed on as
- ☐ Serial Number
- ☐ Express Mail No. , as Serial Number not yet known

(c) ☒ was described and claimed in PCT International Application No. PCT/US99/22520 filed on September 30, 1999 and as amended under PCT Article 19 on (if any), which entered the U.S. national phase as Serial Number 09/806,370.

ACKNOWLEDGEMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37 CFR 1.56(a).

Docket No: 33,383-00
Patent

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventors certificate or of any PCT International application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate of any PCT International application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed.

(d) ☒ No such applications have been filed.
(e) ☐ Such applications have been filed as follows.

NOTE: Where item (c) is entered above and the International Application which designated the U.S. claimed priority, check item (e), enter the details below and make the priority claim.

Earliest Foreign Application(s), if any, filed within 12 months (6 months for Design) prior to this U.S. Application

Country	Application No.	Date of Filing (Day, Month, Year)	Priority Claimed 35 USC 119
PCT	PCT/US99/22520	30 September 1999	

All Foreign Application(s), if any, Filed More Than 12 Months (6 Months for Design) Prior to This U.S. Application)

Docket No: 33,383-00
Patent

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. § 119(E))

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

PROVISIONAL APPLICATION NUMBER	**FILING DATE**
60/102,430	September 30, 1998

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)
(UNDER 35 U.S.C. 120)

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT International filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 USC 120

U.S. Applications		**Status (Check One)**		
U.S. Applications	U.S. Filing Date	Patented	Pending	Abandoned
		☐	☐	☐
		☐	☐	☐
		☐	☐	☐
		☐	☐	☐

Docket No: 33,383-00
Patent

PCT Applications Designating U.S.		
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NO. ASSIGNED (if any)
PCT/US99/22520	September 30, 1999	09/806,370

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As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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Docket No: 33,383-00
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Inventor's Signature _____ Date _____

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Residence : **23371 Morning Rose Drive, Golden, Colorado 80401**
Post Office Address: **23371 Morning Rose Drive, Golden, Colorado 80401**

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Inventor's Signature _____ Date _____

Country of Citizenship: **United Kingdom**
Residence : **12743 East Wyoming Place, Aurora, Colorado 80012**
Post Office Address: **12743 East Wyoming Place, Aurora, Colorado 80012**

Full name of THIRD JOINT INVENTOR: **John H. Eldridge**

Inventor's Signature John H. Eldridge Date 9-12-01

Country of Citizenship: **United States of America**
Residence : **4 Beatrice Cove, Fairport, New York 14450**
Post Office Address: **4 Beatrice Cove, Fairport, New York 14450**

Full name of FOURTH JOINT INVENTOR: **Bruce A. Green**

Inventor's Signature Bruce A. Green Date 09-10-01

Country of Citizenship: **United States of America**
Residence : **49 Northfield Gate, Pittsford, New York 14534**
Post Office Address: **49 Northfield Gate, Pittsford, New York 14534**

Full name of FIFTH JOINT INVENTOR: **Gerald E. Hancock**

Inventor's Signature Gerald E Hancock Date 09-11-01

Country of Citizenship: **United States of America**
Residence : **50 Plains Road, Honeoye Falls, New York 14472**
Post Office Address: **50 Plains Road, Honeoye Falls, New York 14472**

SIGNATURE(S) (continued)

Full name of SIXTH JOINT INVENTOR: **Joel A. Peek**

Inventor's Signature _____ Date _____

Country of Citizenship: **United States of America**
Residence : **115 Moores Court, Brentwood, TN 37027**
Post Office Address: **115 Moores Court, Brentwood, TN 37027**

Docket No: 33,383-00
Patent

COMBINED DECLARATION AND POWER OF ATTORNEY
(Original, Design, Supplemental, Divisional, Continuation,CIP)

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My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

Mutant Cholera Holotoxin as an Adjuvant

SPECIFICATION IDENTIFICATION

the specification of which: (complete (a), (b), or (c))

(a) ☐ is attached hereto.
(b) ☐ was filed on as
☐ Serial Number
☐ Express Mail No. , as Serial Number not yet known
(c) ☒ was described and claimed in PCT International Application No. PCT/US99/22520 filed on September 30, 1999 and as amended under PCT Article 19 on (if any), which entered the U.S. national phase as Serial Number 09/806,370.

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(e) ☐ Such applications have been filed as follows.

NOTE: Where item (c) is entered above and the International Application which designated the U.S. claimed priority, check item (e), enter the details below and make the priority claim.

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Country	Application No.	Date of Filing (Day, Month, Year)	Priority Claimed 35 USC 119
PCT	PCT/US99/22520	30 September 1999	

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(35 U.S.C. § 119(E))

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PROVISIONAL APPLICATION NUMBER	**FILING DATE**
60/102,430	September 30, 1998

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(UNDER 35 U.S.C. 120)

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT International filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 USC 120

U.S. Applications		**Status (Check One)**		
U.S. Applications	U.S. Filing Date	Patented	Pending	Abandoned
		☐	☐	☐
		☐	☐	☐
		☐	☐	☐
		☐	☐	☐

PCT Applications Designating U.S.		
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NO. ASSIGNED (if any)
PCT/US99/22520	September 30, 1999	09/806,370

POWER OF ATTORNEY

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☒ Customer Number: 25291 Bar Code:

☐ Attached as part of this declaration and power of attorney is the authorization of the above-named attorney(s) to accept and follow instructions from my representative(s).

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Tel. No. (845) 602-4636

Docket No: 33,383-00
Patent

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Inventor's Signature _____ Date _____

Country of Citizenship: **United States of America**
Residence : **23371 Morning Rose Drive, Golden, Colorado 80401**
Post Office Address: **23371 Morning Rose Drive, Golden, Colorado 80401**

Full name of SECOND JOINT INVENTOR: **Michael G. Jobling**

Inventor's Signature _____ Date _____

Country of Citizenship: **United Kingdom**
Residence : **12743 East Wyoming Place, Aurora, Colorado 80012**
Post Office Address: **12743 East Wyoming Place, Aurora, Colorado 80012**

Full name of THIRD JOINT INVENTOR: **John H. Eldridge**

Inventor's Signature _____ Date _____

Country of Citizenship: **United States of America**
Residence : **4 Beatrice Cove, Fairport, New York 14450**
Post Office Address: **4 Beatrice Cove, Fairport, New York 14450**

Full name of FOURTH JOINT INVENTOR: **Bruce A. Green**

Inventor's Signature _____ Date _____

Country of Citizenship: **United States of America**
Residence : **49 Northfield Gate, Pittsford, New York 14534**
Post Office Address: **49 Northfield Gate, Pittsford, New York 14534**

Full name of FIFTH JOINT INVENTOR: **Gerald E. Hancock**

Inventor's Signature _____ Date _____

Country of Citizenship: **United States of America**
Residence : **50 Plains Road, Honeoye Falls, New York 14472**
Post Office Address: **50 Plains Road, Honeoye Falls, New York 14472**

SIGNATURE(S) (continued)

Full name of SIXTH JOINT INVENTOR: **Joel A. Peek**

Inventor's Signature Joel Peek Date Sept. 6, 2001

Country of Citizenship: **United States of America**
Residence : **115 Moores Court, Brentwood, TN 37027**
Post Office Address: **115 Moores Court, Brentwood, TN 37027**

SEQUENCE LISTING

<110> American Cyanamid Company

<120> Mutant Cholera Holotoxin As An Adjuvant

<130> 33383-00 PCT

<140> 60/102,430
<141> 1998-09-30

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09806370 .100301

33,383-00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Randall K. Holmes et al.

Serial No. : 09/806,370

Filed : September 30, 1999

For : Mutant Cholera Holotoxin as an Adjuvant

Examiner : To be Assigned

Group Art Unit : To be Assigned

Confirmation No.: 8568 Customer No.: 25291

October 3, 2001

BOX MISSING PARTS
Hon. Commissioner for Patents
Washington, D.C. 20231

STATEMENT UNDER 37 C.F.R. 1.821(g)

Sir:

I hereby certify that the content of the paper copy required by 37 C.F.R. 1.821(c) and the computer readable form required by 37 C.F.R. 1.821(e) of the Sequence

~~~~~~

CERTIFICATION UNDER 37 C.F.R. 1.10

I hereby certify that this paper and the documents referred to as enclosed therein are being deposited with the United States Postal Service on the date written below in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number ET334776318US addressed to the Commissioner for Patents, Washington, D.C. 20231.

10/3/01
Date

Alan M. Gordon
Alan M. Gordon

~~~~~~

Listings for the above-captioned U.S. national stage application based on PCT international patent application number PCT/US99/22520 as filed are the same, that is, the information recorded in computer readable form is identical to the written Sequence Listing, and the submission contains no new matter.

Respectfully submitted,

Alan M. Gordon
Registration No. 30,637
American Home Products Corp.
Patent Law Department
Five Giralda Farms
Madison, NJ 07940
(845) 602-4636
Attorney for Applicants

L:\amend1ag\1agrj23.doc

SEQUENCE LISTING

<110> American Cyanamid Company

<120> Mutant Cholera Holotoxin As An Adjuvant

<130> 33383-00 PCT

<140> 60/102,430
<141> 1998-09-30

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